THE EFFECTS OF PRENATAL AND POSTNATAL CONTEXTS ON PHYSIOLOGY, BEHAVIOUR AND SURVIVAL OF REINTRODUCED GREY PARTRIDGES (*PERDIX PERDIX*)

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SUMMARY

Phenotypic variation emerges from the interplay of genetic and environmental factors. Investigating the factors that drive phenotypic expression is essential to understanding evolutionary processes and should enable more efficient conservation strategies.

Prenatal environmental conditions during early development can affect postnatal phenotypes through parental hormones (parental effects) and likewise postnatal developmental trajectories respond to environmental triggers. The phenotypic variation induced by prenatal and postnatal factors can be adaptive, i.e. it can increase fitness in a specific environment. As a consequence, experimental combinations of different genetic, prenatal and postnatal contexts could yield phenotypes best able to thrive in a wild, post-release environment.

I investigated how grey partridges (*Perdix perdix*) of two different strains (wild and domesticated) translated phases of prenatal and postnatal predictable or unpredictable food supply into phenotypic variation. Then I explored the survival differences between the eight experimental groups (strain x prenatal x postnatal) after release into the wild. I also considered family and covey effects, i.e. the resemblance of phenotypic traits and survival between full siblings and social group mates and I determined the individual flexibility of two important phenotypic traits, behaviour and corticosterone, a primary metabolic and stress related hormone in birds.

Compared to the domesticated strain, grey partridges of the wild strain showed stronger immune indices, higher oxidative stress resistance and a more pronounced short-term (up to 250 seconds) and medium-term (around 30 min) corticosterone response to a stressor (Chapters 1 and 2). There was some evidence that an unpredictable prenatal food supply induced adaptive parental effects. However, these effects were modulated differently by strains and were probably inconsistent between the two study years. When considering the study year 2010 only, wild strain birds exhibited a lower corticosterone response to stress following an unpredictable prenatal food supply (Chapter 1). When considering both study years an unpredictable prenatal food supply accelerated the developmental trajectory of the corticosterone response to stress in the domesticated strain (Chapter 2).

Irrespective of strain and prenatal feeding scheme, unpredictable postnatal food supply enhanced part of the innate and the adaptive immune system and it also increased post-release survival (Chapters 1 and 4). Furthermore, survival was related to behaviour, as defined by three commonly used behavioural tests. Survival increased with decreasing

boldness and was highest at intermediate levels of sociability (Chapter 3). Coveys, i.e. social groups apparently affected individual phenotypes on levels from the circulation of corticosterone to behaviour and ultimately survival (Chapters 1 to 4).

Corticosterone appears to be an important indicator of an individual's state. High corticosterone was related to passive and shy behaviour (Chapter 3). Longer acclimatisation time at the release site was related to lower levels of corticosterone and partly enhanced survival after release (Chapter 5).

Grey partridges showed a substantial ability to translate prenatal and postnatal environmental conditions into (adaptive) phenotypic differences and corticosterone played a crucial role at the interface of the internal physiological milieu with the external environment. Importantly, the captive environment offers considerable potential to prepare release candidates for the post-release environment. Controlling the postnatal feeding conditions was simple and practical. Its positive effects on the immune system and on post-release survival might well be found in other species which could ultimately aid animal reintroductions and conservation in general. Finally, the substantial covey effects underlines the high sociality in this species and suggest that strategic manipulation of the social group prior to release could prove useful to enhance re-introduction success in gregarious species.

ZUSAMMENFASSUNG

Phänotypische Variabilität entsteht aus dem Zusammenspiel von genetischen und Umweltfaktoren. Um evolutive Prozesse zu verstehen und die Natur effizient zu schützen, ist es zentral, die Faktoren zu verstehen, welche die phänotypische Ausprägung steuern.

Der Phänotyp eines Individuums kann durch die pränatale Umwelt über (hormonelle) Elterneffekte beeinflusst werden und, auch postnatale Umwelteinflüsse können die Ausprägung des Phänotyps steuern. Die phänotypische Variabilität, welche durch solche prä- und postnatale Faktoren induziert wird, kann adaptiv sein, d.h. sie kann unter gewissen Umweltbedingungen die biologische Fitness erhöhen. Daraus ergibt sich, dass verschiedene genetische, pränatale und postnatale Faktoren so kombiniert werden könnten, dass Phänotypen entstehen, die optimal an ein Überleben nach dem Aussetzen in die freie Wildbahn angepasst sind.

Ich untersuchte, wie Rebhühner (*Perdix perdix*) aus zwei verschiedenen Zuchtlinien (wild und domestiziert) Zeitabschnitte mit vorhersehbarem oder unvorhersehbarem Futterzugang vor und nach der Geburt in phänotypische Variation umsetzten. Anschliessend untersuchte ich, ob es zwischen den acht experimentellen Gruppen (Genetische Linien x pränatale x postnatale Behandlung) Überlebensunterschiede nach dem Freisetzen in die freie Wildbahn gab. Dabei untersuchte ich auch die Ähnlichkeit von Geschwistern (genetische Verwandtschaft) und von Mitgliedern derselben sozialen Gruppe im Phänotyp und im Überleben. Zudem bestimmte ich die individuelle Flexibilität zweier wichtiger phänotypischer Merkmale, des Verhaltens und des Kortikosterons. Kortikosteron ist ein wichtiges metabolisches Hormon der Vögel involviert in der physiologischen Antwort auf Stress.

Im Vergleich zur domestizierten Linie zeigten Vögel der Wildlinie stärkere Immunantworten, höhere Resistenz gegen oxidativen Stress und eine ausgeprägtere Kortikosteronantwort auf eine Störung (Kapitel 1 und 2). Es gab Hinweise darauf, dass eine unvorhersehbare Nahrungsverfügbarkeit vor der Geburt adaptive Elterneffekte verursachen könnte. Diese Effekte waren aber unterschiedlich in den beiden Zuchtlinien und möglicherweise inkonsistent zwischen den beiden Studienjahren. Wenn nur das Jahr 2010 betrachtet wurde, zeigten Vögel der wilden Zuchtlinie eine tiefere Kortikosteronantwort auf Stress, wenn sie unter pränatal unvorhersehbarer Nahrungsverfügbarkeit gehalten wurden (Kapitel 1). Wenn beide Studienjahre berücksichtigt wurden, beschleunigte die pränatal

unvorhersehbare Nahrungsverfügbarkeit die Entwicklung der Kortikosteronantwort bei der domestizierten Zuchtlinie (Kapitel 2).

Unabhängig von der Zuchtlinie oder der pränatalen Nahrungsverfügbarkeit erhöhte postnatal unvorhersehbare Nahrungsverfügbarkeit Teile der angeborenen und der angeeigneten Immunantwort und sie erhöhten auch das Überleben nach der Freilassung (Kapitel 1 und 4). Das Verhalten, gemessen mit drei häufig verwendeten Verhaltenstests, korrelierte ebenfalls mit dem Überleben. Das Überleben war am höchste bei Vögeln mit dreistem Verhalten und mittlerer Geselligkeit (Kapitel 3). Die Ketten (soziale Gruppen) beeinflussten die individuellen Phänotypen auf verschiedenen Ebenen, angefangen bei den Kortikosteronwerten im Blut bis hin zum Verhalten und Überleben (Kapitel 1 und 4). Kortikosteron scheint den Zustand eines Individuums gut widerzuspiegeln. Hohes Kortikosteron korrelierte mit eher passivem und scheuem Verhalten (Kapitel 3). Längere Akklimatisationszeiten vor der Aussetzung gingen einher mit tieferem Kortikosteron und teilweise erhöhtem Überleben nach der Freilassung (Kapitel 5).

Rebhühner konnten pränatale und postnatale Umweltbedingungen in (adaptive) phänotypische Variation umsetzten und Kortikosteron scheint eine Schlüsselposition inne zu haben an der Schnittstelle zwischen interner (physiologischer) und externer Umwelt. Bemerkenswert ist, dass die Tiere in Gefangenschaft auf ein Überleben in freier Wildbahn vorbereitet werden können. Die Kontrolle der Fütterungsbedingungen während der Kükenaufzucht ist mit geringem Aufwand verbunden, und die daraus folgenden positiven Effekte auf das Immunsystem und das Überleben könnten auch bei anderen Arten beobachtet werden. Dies könnte die Erfolgschancen von Wiederansiedlungen erhöhen und somit dem Erhalt von bedrohten Arten generell dienen. Die beachtlichen Ketteneffekte unterstreichen die Wichtigkeit der sozialen Gruppe bei dieser Art. Gezielte Manipulationen der Gruppe vor der Freilassung könnten es erlauben, den Wiederansiedlungserfolg sozialer Arten zu erhöhen.

GENERAL INTRODUCTION

Variability is an essential feature of life or to use the words of Charles Darwin (1859): “unless profitable variations do occur, natural selection can do nothing”. Ultimately, high phenotypic variation within populations facilitates survival and the reproduction of at least some individuals even under severe environmental conditions. On the other hand, organisms that cannot cope with given conditions or cannot adapt to changing environments will not persist into to the future (Mayr 2003). Individual phenotypes (and thus variation among them) emerge from the interplay of genes and the environment. The heritable genetic blueprint sets the principal body plan and determines the evolutionary potential over generations. This potential and its consequences for the phenotype can be seen, for example, when comparing domesticated animals with their wild ancestors (Trut 1999; Driscoll, Macdonald & O'Brien 2009; Wiener & Wilkinson 2011). Alongside these processes playing out over generations, environmental conditions often change considerably within short timescales, i.e. between parents and their offspring or within the lifetime of a single generation. Therefore, being able to adequately respond to such predictable and unpredictable short-term environmental fluctuations could convey a selective advantage (Piersma & Drent 2003). In fact, animals do show phenotypic plasticity, i.e. the expression of a single genotype differs depending on environmental conditions and even mature organisms are flexible to some degree, i.e. show reversible variation in phenotypic traits (Piersma & Drent 2003). Illustrative examples of the plasticity and flexibility of phenotypes can be found in animals that undergo life-cycle stages, i.e. adjust their phenotypes to predictable cyclic (seasonal) changes in the environment (Piersma & Lindström 1997) or are evident when comparing wild animals in their natural environment to conspecifics in captivity (Mason *et al.* 2013). Understanding the factors which govern phenotypic variability, i.e. the interplay of genetic factors, plasticity and flexibility is crucial to predict a species’ response to the current and future environment and hence to define efficient species conservation measures.

Environmental conditions prevailing during the prenatal and early postnatal development can induce irreversible modifications in the phenotype which could prime individuals for the demands of the future (Love, McGowan & Sheriff 2013). The translation of prenatal conditions to the offspring can be achieved by parental effects, i.e. the transfer of developmental resources from parents to their offspring, which in turn influence genetically inherited components of the phenotype (Badyaev & Uller 2009). For example, subjecting great tit (*Parus major*) mothers to high predation risk during ovulation resulted in

lower levels of testosterone in the egg yolk and ultimately in a lower body size and increased wing growth of their offspring as compared to a control group. Hence, mothers seem to have translated high predation risk during ovulation into phenotypic adaptations of their offspring, i.e. small size and low mass but increased wing size at maturity, all of which might have enhanced their ability to evade predators (Coslovsky & Richner 2011; Coslovsky *et al.* 2012). While such transgenerational effects can be valuable means of adaptation, they also impose the risk of phenotypic mismatches if prenatal triggers do not reliably predict the postnatal conditions (Raubenheimer, Simpson & Tait 2012).

Maternal hormones are primary candidates for the role of mediators of parental effects (Meylan, Miles & Clobert 2012). Indeed, experimental manipulation of steroid hormones such as androgens or glucocorticoids can affect developmental trajectories in mammals and birds (Viltart & Vanbesien-Mailliot 2007; Uller 2008; Henriksen, Rettenbacher & Groothuis 2011). Investigating glucocorticoid mediated maternal effects in birds is especially appealing for three reasons: First, in contrast to the sex steroids (progestins, androgens, oestrogens), which are mainly produced within the ovarian follicles, glucocorticoids are produced distantly by the maternal adrenal cortex and have to be transported and deposited into the egg via the circulation (Groothuis & Schwabl 2008). Hence, glucocorticoids present in the egg at an early stage can only have maternal sources. Second, eggs are built within relatively narrow time frames (around 4–14 days depending on the species), limiting the time window when maternal hormone deposition into the egg can take place. Finally, after oviposition, embryos develop within the egg, which does constitute a sealed compartment separated from the mother. Hence, eggs can easily be relocated and incubated without the interference of other maternal effects (e.g. maternal incubation behaviour) under defined (artificial) conditions.

Glucocorticoids, i.e. corticosterone in birds, orchestrate the expression of many physiological and behavioural traits including immunity, energetic metabolism and parts of the physiological response to acute stress (Sapolsky, Romero & Munck 2000; Romero 2004). During early life glucocorticoids have important organizational functions (e.g. they affect brain development) and hence they show substantial capacity to convey parental effects (Sapolsky & Meaney 1986; Henriksen *et al.* 2011; Love *et al.* 2013). In adulthood circulating levels of glucocorticoids vary largely between species and among individuals and they are paradigmatic examples for (reversible) phenotypic flexibility since they show pronounced

variability even within individuals (Williams 2008). As such, investigating how glucocorticoids interact with the environment during different life stages and how these interactions affect phenotypic expression is highly intriguing and likely to produce a payoff that could eventually enable us to engineer phenotypes for conservation purposes (Meylan *et al.* 2012).

Disentangling the impacts of different sources of phenotypic variation is difficult since factors that induce them often co-vary. For example, the adult size of offspring is strongly affected by genetic factors, i.e. it correlates with the size of the mother and father, but also depends on the nutritional supply to the offspring (Boag & van Noordwijk 1987). Likewise, the physiological response to stress is partly heritable (Odeh, Cadd & Satterlee 2003) but also depends on current body condition (Kitaysky *et al.* 2001; Bonier *et al.* 2009) and social environment (Creel *et al.* 2013). To tease apart different sources of phenotypic variation the prenatal, postnatal, social and non-social environment has to be controlled as thoroughly as possible. By readily accommodating these requirements, bird models are again appealing on account of the characteristics mentioned above. In particular, precocial bird species offer a distinct advantage since they are not necessarily dependent on postnatal parental care and can thus be reared under relatively well controlled postnatal conditions.

The grey partridge (*Perdix perdix*) is a precocial, ground-dwelling wild fowl species which originally inhabited open grassland and has been widely distributed across Europe for at least the past two million years (Potts 2012). The nominate sub-species *P. p. perdix* ranging from middle to northern Europe is sedentary and thus particularly dependent on suitable microhabitats within its home range throughout the year. This makes it a sensitive indicator of local habitat quality and an umbrella species (Potts 2012). What is biologically especially remarkable is the large clutch size of the species (between 15 and 18 but can even exceed 20) (Glutz von Blotzheim, Bauer & Bezzel 1994; Potts 2012) and its high sociability. Indeed, after fledging, families form coveys, i.e. social groups, which stay together throughout the year and only disintegrate with the dawn of the subsequent spring when pair formation for the next breeding season starts. Coveys are sometimes joined by solitary adult birds, often widowed males, which confirms the importance of social groups for this species (Watson, Aebischer & Cresswell 2007; Tillmann 2009a; Tillmann 2009b). The sedentary way of life and the high vulnerability to predation imply that grey partridge behaviour is subtly adjusted to the prevailing conditions, including climate and predator presence (Glutz von Blotzheim *et al.* 1994; Potts 2012). For example, feeding activity is highest in the morning

and evening hours and is mostly performed in or close to cover, primarily field margins, which probably reduces the likelihood of a lethal raptor attack. By contrast, during the night grey partridges roost in open areas at a safe distance from the field margins where nocturnal predators search for food (Tillmann 2009b).

The fate of the grey partridge has been strongly intertwined with human history since at least 10'000 years ago when human settlements and agricultural areas started to emerge. At that time, the species was able to adapt to increasing human presence and even profited from the cultivation of land and grain. On the other hand, throughout this period it has been a common and valued game species, yielding enormous annual hunting bags (Potts 2012) and also leaving its imprint in human arts and mythology (Jenny, Holzgang & Zbinden 2005). To this day, the grey partridge's ability to cope with human proximity is impressively documented by their survival alongside urban areas of high population density (Salek *et al.* 2004). However, in the arable core habitats grey partridge numbers started to dwindle drastically throughout Europe as agricultural practices were hugely intensified and the use of herbicides became widespread in the second half of the twentieth century. The decline exceeded 90% over the whole of the twentieth century (Potts 2012) and is still on-going today (the European bird census council documents an 80% decline between 1980 and 2008 in Europe; URL www.ebcc.info). During the 1990s the Swiss Ornithological Institute aimed to halt the decline in Switzerland by restoring habitats in two regions, the Klettgau (8°31'E, 47°42'N) in the canton of Schaffhausen and the Champagne genevoise in the canton of Geneva (6°04'E, 46°15'N). These two regions were the last natural strongholds of grey partridges in Switzerland. Despite improvements to habitats relevant for grey partridges, the species had become virtually extinct by the turn of the millennium, which prompted the Swiss Ornithological Institute to start a reintroduction project (Jenny *et al.* 2005; Buner *et al.* 2005).

Animal introductions and especially introductions of galliforms have a long history in Western Europe reaching back at least into the middle ages (Potts 2012). Traditionally, hunting, i.e. diversifying and restocking hunting bags, has been the main purpose of introductions. Nowadays, reintroducing animals into parts of the native range from which they have disappeared is becoming an increasingly popular tool in conservation biology (Armstrong & Seddon 2008; Seddon, Strauss & Innes 2012). However, animal reintroductions are controversial and often criticised for inefficient use of conservation

funds. First, they could imply an 'exploit now and fix later ' mentality, i.e. rather than preventing initial population declines, animals can 'simply' be bred in captivity and reintroduced after local extinction. Second, research within reintroduction projects is often based on monitoring data obtained ad hoc, rather than first formulating *a priori* questions and then defining appropriate monitoring methods to answer them. Consequently, we still lack, but urgently need, a more thorough understanding of why many reintroduction attempts fail (Armstrong & Seddon 2008).

One important factor influencing reintroduction success is the phenotypic quality of release candidates. For example, animals originating from wild populations fare better after release than animals from populations which had been kept and bred in captivity for many generations (Fischer & Lindenmayer 2000). Indeed, captive breeding has been shown to affect physiology and behaviour in ways detrimental to fitness in the wild (McDougall *et al.* 2006). Hence, wild animals have physiological and behavioural attributes appropriate for wild environments, but often it is impossible to obtain enough individuals from sustainable wild populations for financial, ethical and logistical reasons. An alternative approach can be to prepare the phenotypes of release candidates from captive populations to better suit the demands of the post-release environment (Swaisgood 2010). Typically, pre-release preparatory measures have focused on behaviour (Griffin, Blumstein & Evans 2000). On the other hand, the importance of morphological and physiological traits in determining post-release survival has repeatedly been acknowledged (Putaala & Hissa 1995) but rarely actively tackled experimentally.

In conclusion, using defined sets of prenatal and postnatal rearing conditions should ensure phenotypic variability and some of these varieties might be well-adapted to the post-release environment. Using appropriate sets of perinatal conditions could even increase the post-release fitness prospects of captive populations to match those of populations bred in the wild.

This thesis

In this thesis I investigate how the genetic background, the prenatal parental environment and the postnatal early-life environmental conditions shape the phenotype of grey partridges. Then I explore how natural selection acts upon the phenotypic varieties in the wild. The thesis was integrated into a species conservation and recovery project, namely the

reintroduction of grey partridge into Switzerland (Jenny *et al.* 2005), which facilitated two important research goals. First, it made it possible to consider (and partly control) all the (ontogenetic) stages from the birds' genetic and parental origin throughout their rearing and social environment up to and including their release conditions. Second, it meant that scientific questions which were important from a purely research perspective could be tackled methodically in ways which potentially have direct implications for applied conservation. In other words, I investigated how prenatal and postnatal contexts affected phenotypic expression and whether birds can be prepared for the post-release environment by the appropriate adjustment of these contexts. To investigate these questions I used grey partridges of two captive strains. The first strain was established from wild pairs captured from a sustainable population on a large estate in eastern England. Eggs supplied by parental pairs of the third captive generation of these partridges gave rise to what is subsequently called the 'wild strain'. The second strain consisted of grey partridges which had been kept and bred in captivity for at least 30 generations and is thus subsequently called the 'domesticated strain'. The two strains were expected to vary considerably in physiology and behaviour due to random genetic changes and/or adaptations to captivity (Price 1999; Keller et al. 2012). We subjected parental pairs of the two strains and their offspring to periods of unpredictable food supply or *ad libitum* conditions which eventually provided the eight treatment groups (strain x prenatal x postnatal) with which I worked.

I started my analysis at the physiological core of the individuals, then expanded my view to include behaviour and finally considered the consequences of phenotypic variability for survival. In the **first chapter** I examine how parts of the immune system, the physiological stress axis (HPA axis) and the (anti)oxidative system work together and whether they differed between strains and were affected by prenatal and postnatal feeding conditions. These physiological systems are ubiquitous in vertebrate life. They have profound implications for many other phenotypic traits and directly affect an organism's fitness (e.g. Sapolsky *et al.* 2000; Wingfield & Sapolsky 2003; Dowling & Simmons 2009).

Hormones have essential organizational functions during development and activate many downstream processes throughout life. In particular, the excretion of corticosterone by the HPA axis merits attention since adequate physiological responses to stressors are important in coping with predictable and unpredictable challenges throughout life. The **second chapter** thus comprises an in-depth analysis of the effects of strains and perinatal

treatments on postnatal developmental trajectories of baseline and stress-induced levels of corticosterone. In addition, repeated corticosterone measures within individuals allowed their repeatability to be quantified.

Behaviour is an apparent phenotypic characteristic of every animal. We commonly ascribe distinct behavioural types such as bold or shy personalities to individuals in a variety of taxa. There is a lively scientific debate about the proximate mechanisms, i.e. hormonal correlates of behaviours and personalities and their fitness consequences. In the **third chapter** I contribute to this discussion and examine the effects of strain, prenatal and postnatal treatments and other important factors on behavioural traits. In addition, correlations between behavioural traits and the relationships between corticosterone levels, behaviour and post-release survival are part of this integrative chapter.

The **fourth chapter** is dedicated to the effects of strain, perinatal treatments and additional factors on post-release survival. I used Bayesian multistate capture-recapture models to jointly analyse data obtained by radio-telemetry, visual observations of marked individuals and recoveries of dead birds. This flexible framework allowed me to consider a large amount of different data in one analysis and let me make inferences not only on probabilities of survival between the strains and treatments but also on encounter and recovery rates of different groups.

The final **fifth chapter** takes on a specific open question of animal reintroductions. Acclimatisation time at the release site prior to release can positively affect post-release survival (Bernardo *et al.* 2011). Thus, the ability to cope with translocation stress could actually be central but has hardly been investigated. In her Master's thesis Nina Keller investigated the impact of pre-release transportation and handling procedures on corticosterone levels of grey partridges. Also, we jointly explored whether acclimatization time on-site can positively influence stress hormone levels and post-release survival.

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CHAPTER 1

The impact of pre- and post-natal contexts on immunity, glucocorticoids and oxidative stress resistance in wild and domesticated grey partridges

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Summary

1. Genetic background, prenatal and postnatal early-life conditions influence the development of interconnected physiological systems and thereby shape the phenotype. Certain combinations of genotypes and pre- and postnatal conditions may provide higher fitness in a specific environmental context.

2. Here, we investigated how grey partridges *Perdix perdix* of two strains (wild and domesticated) cope physiologically with pre- and postnatal predictable versus unpredictable food supply. Food unpredictability occurs frequently in wild environments and requires physiological and behavioural adjustments.

3. Well-orchestrated and efficient physiological systems are presumably more vital in a wild environment as compared to captivity. We thus predicted that wild strain grey partridges have a stronger immunity, glucocorticoid (GC) stress response and oxidative stress resistance (OSR) than domesticated birds which have undergone adaptations to captivity. We also predicted that wild strain birds react more strongly to environmental stimuli and, when faced with harsh prenatal conditions, are better able to prepare their offspring for similarly poor postnatal conditions than birds of domesticated origin.

4. We found that wild strain offspring were physiologically better prepared for stressful situations as compared to the domesticated strain. They had a high GC stress response and a high OSR when kept under predictable food supply. Wild strain parents reacted to prenatal unpredictable food supply by lowering their offspring's GC stress response which potentially lowered GC-induced oxidative pressure. No such pattern was evident in the domesticated birds.

5. Irrespective of strain and prenatal feeding scheme, postnatal unpredictable food supply boosted immune indices, and GC stress response was negatively related to antibody response in females and to mitochondrial superoxide (mSO) production.

6. Wild strain grey partridge showed fitness-relevant physiological advantages and appeared to prepare their offspring for the prospective environment. Negative relationships between GC stress response, immunity and oxidative indices imply a pivotal role of an organism's oxidative balance and support the importance of considering multiple physiological systems simultaneously.

Keywords: oxidative stress resistance, glucocorticoid stress response, immunocompetence, mitochondrial superoxide production, maternal effects, domestication effects, physiological networks, trade-off, animal re-introduction, oxidative balance

Introduction

Investigating the sources and consequences of phenotypic variation is central for understanding the evolution of life-history strategies and important for the conservation of threatened species (Ricklefs & Wikelski 2002). Three major factors contribute to shape the phenotype, namely the genetic background, the prenatal parental environmental and the postnatal early-life environmental conditions. These factors can interact with each other and provide the physiological framework on which phenotypic variation is based (e.g. Love *et al.* 2008; Henriksen, Rettenbacher & Groothuis 2011; Cohen *et al.* 2012).

Genetic variation provides the raw material for selection and partly determines the evolutionary potential of a species. Considering the genetic origin is especially important in the context of animal reintroductions using captive populations. Reduced genetic diversity (Keller *et al.* 2012) and adaptations to captivity which are detrimental in the wild (Frankham 2008) could compromise the success of animal reintroductions using captive stocks (Fischer & Lindenmayer 2000).

In addition to the genetic make-up, the phenotype is shaped by parental effects (Mousseau & Fox 1998; Breuner 2008; Love & Williams 2008a; Coslovsky & Richner 2011). Prenatal maternal effects (i.e. during the egg laying period or gestation) can be seen as a mechanism to adapt the offspring to the prospective environment the mother experienced

before or during pregnancy/gestation or egg laying (Mousseau & Fox 1998; Love & Williams 2008b). This may be a direct consequence of limited resources which, for example, increase maternal GCs which in turn are transferred proportionally to the embryo and prepare it to harsh conditions (Breuner 2008; Love & Williams 2008a). It may also be a regulated transfer of hormones (e.g. androgens) depending on environmental and maternal conditions (Groothuis & Schwabl 2008).

Postnatal early-life conditions, the third major factor shaping the phenotype, have been shown to induce lasting effects on an individual's phenotypic expression and can consequently affect fitness (Metcalfe & Monaghan 2001; Tschirren *et al.* 2009). Thus, a certain combination of genetic background, prenatal and early life conditions may shape the offspring's phenotype to suit a specific environment.

Phenotypic variation emerges from a complex interplay of physiological systems and the environment (Cohen *et al.* 2012). These physiological interactions and environmental constraints may entail physiological trades-offs which in turn might constrain phenotypic expression (Ricklefs & Wikelski 2002). Physiological systems that were shown to be affected by environmental conditions include the GC stress response (the excretion of GCs as a response to stress by the hypothalamic-pituitary-adrenal axis) (Love & Williams 2008b) and the immune system (Tschirren *et al.* 2009). The GC excreting system and the immune system are interconnected in such a way that high levels of GCs often down-regulate immune reactivity (Sapolsky, Romero & Munck 2000; but see Martin 2009). Thus, there appears to be a trade-off between immunity and GC stress response in that a high activity of one system constrains the activity of the other. An associated cost of both, the immune system and GC stress response, is the production of reactive oxygen species (ROS) (Knight 2000; Costantini & Møller 2009). ROS are highly reactive molecules which are ubiquitous in aerobic organisms (Dowling & Simmons 2009). While ROS support vital functions such as pathogen destruction in an immune response (Knight 2000), they also pose an oxidative threat that has to be tightly controlled in order to minimize indiscriminate damage to the cells (Monaghan, Metcalfe & Torres 2009). Therefore, an organism possesses an arsenal of enzymatic and non-enzymatic antioxidants that maintains oxidative homeostasis under normal conditions. However, if the oxidative load exceeds the capacity of the antioxidant system the balance is shifted towards ROS resulting in oxidative stress and its malign consequences to the host cell. In recent years ROS have repeatedly been suggested as

important players in the evolution of life history strategies (Dowling & Simmons 2009; Monaghan *et al.* 2009; Metcalfe & Alonso-Alvarez 2010) and they could constrain the expression of different phenotypes. Because the production of ROS can increase drastically within a short time-scale (e.g. due to intense physical activity, immune challenge, GC stress response (Knight 2000; Sachdev & Davies 2008; Costantini, Marasco & Møller 2011) there must normally be an excess of antioxidant capacity as a contingency buffer (Monaghan *et al.* 2009), which is called OSR. Thus, OSR is the remaining current capacity to prevent oxidative stress which integrates past and present oxidizing events with regard to all antioxidants available (Lesgards *et al.* 2002; Stocker *et al.* 2003). Another important index of the oxidative balance is mSO production. Mitochondria are responsible for the majority of ROS generation (Balaban, Nemoto & Finkel 2005) and superoxide is the principal ROS produced (Turrens 1997; Mukhopadhyay *et al.* 2007).

In the present study we controlled prenatal and early-life food availability of two strains of grey partridges (*Perdix perdix*) (Fig. 1). Food availability is a crucial factor for wild birds, affecting for example timing of reproduction (Schoech & Hahn 2008) or offspring sex ratio (Merkling *et al.* 2012). Unpredictable or restricted access to food can affect immune function (Alonso-Alvarez & Tella 2001) and provoke physiological changes such as weight loss and/or increased levels of circulating GCs in birds (Lynn *et al.* 2010). Circulating levels of GCs in female birds during egg-laying can be correlated with GC levels in the egg and affect the offspring's stress axis (Love, McGowan & Sheriff 2012). GCs could also affect the offspring by modulating levels of other egg hormones (e.g. high circulating maternal GCs entailed lower levels of yolk progesterone and testosterone; (Henriksen, Groothuis & Rettenbacher 2011).

We took advantage of a re-introduction project of grey partridges into Switzerland (Jenny, Holzgang & Zbinden 2005) which used recent descendants of wild birds *versus* birds bred in captivity for over 30 generations. The two strains were predicted to differ by the degree of domestication. By combining the two strains with pre- and postnatal food treatments we created eight experimental groups. We determined the impact of strain, pre- and postnatal food availability on indices of the innate and adaptive immune system, on OSR, on mSO production and on the GC stress response. These physiological systems are interconnected and all represent considerable intrinsic sources of ROS which must be balanced (Costantini & Møller 2009; Stier *et al.* 2009; Costantini *et al.* 2011). Thus, we

investigated whether there are correlations between the systems which may be indicative of trade-offs in regard to oxidative balance.

Our first aim in this study was to investigate the impact of moderate stress (experienced as unpredictable food supply during crucial time frames) prenatally and postnatally early in life on important physiological traits that underlie phenotypic variation. We were especially interested in potential multiplicative effects of pre- and postnatal treatment in the two strains. The second aim was to examine the interconnected nature of the physiological traits with regard to their oxidative potential.

We predicted that wild strain grey partridges have a stronger immunity, GC stress response and OSR than domesticated birds which have undergone adaptations to captivity (Price 1999). We hypothesized that unpredictable food supply represented a latent challenging situation which might provoke physiological adaptations. Mothers experiencing unpredictable food supply might prepare their offspring to the expected demanding environmental conditions while unprepared offspring (i.e. offspring that was not prenatally subjected to unpredictable food supply) might suffer from an inappropriate physiological setup (Price 1999; Breuner 2008). The OSR could play an important role since stress physiology, immunity and mitochondrial activity represent intrinsic sources of ROS. To our knowledge this is one of the first studies trying to manipulate three important sources of phenotypic variation and characterising their consequences on important physiological traits in a non-model species.

Materials and methods

Origin of birds

The grey partridge is a ground-dwelling, gamebird species typically inhabiting farmland areas. They have precocial young, are seasonally monogamous and lay normally one clutch per year. In 2010 grey partridge eggs were obtained from a breeder in the UK (Perdix Wildlife Solutions, Warwickshire UK). Eggs originated from two captive strains. For the first strain, male and female grey partridges were captured from a sustainable wild population on a large wild game shooting estate in eastern England. Female offspring from these wild pairs were mated with males captured in the wild the consecutive spring. Offspring of this semi-wild strain were subjected to prenatal treatment (see below) and produced part of the eggs for our study. Thus, these birds are considered to be genetically similar to the wild

population (subsequently called wild strain). The second strain consisted of birds that were kept in captivity for at least 30 generations (one generation per year) without adding new wild birds (subsequently called domesticated strain). The captive environment was believed to have caused random genetic changes and/or adaptations to captivity as they occur during the domestication process (Price 1999; Keller *et al.* 2012). A pilot study found significant differences in polymorphic satellite markers between the two strains, but no signs of inbreeding in the domesticated strain (B. Homberger, unpublished data). During winter wild and domesticated birds were held in two separate aviaries adjacent to each other on the same farm in the UK (Perdix Wildlife Solutions, Warwickshire UK).

Experimental procedure

When mating season started birds were allowed to freely choose their mate from the flock with which they spent the winter. Twenty five pairs of wild and 25 pairs of domesticated grey partridges where then placed into separate outdoor aviaries (3 x 3 x 1.5 m) and randomly assigned to either predictable or unpredictable food supply. In the predictable food supply scheme (12 pairs of each strain) food was available *ad libitum* 24 h a day. In the unpredictable food supply scheme (13 pairs of each strain) food was removed during four hours a day at randomly differing time points (between 8 am and 8 pm) in accordance with the UK regular feeding schemes, while during the remaining time food was available *ad libitum*. The unpredictable feeding scheme began on 5 April 2010, i.e. one week before egg-laying began and lasted throughout the entire egg-laying period until the last series of eggs was transported to Switzerland at the end of June 2010. Grey partridge hens laid 42 eggs on average (no significant difference between feeding schemes). We found that eggs of parents subjected to prenatal unpredictable food supply had a lower progesterone concentration in yolk than eggs of parents subjected to prenatal predictable food supply, while GC and testosterone levels were similar in the two groups of eggs (B. Homberger, unpublished data). This suggests that the food supply scheme altered hormonal maternal effects.

Eggs were collected daily from the aviaries and transported weekly by airplane to Switzerland. They were artificially incubated at the Swiss Ornithological Institute facilities in Sempach. On the hatching day chicks were individually marked with colour rings and assigned to indoor aviaries (200 x 80 x 80 cm) in Sempach with approximately 30 birds of the same hatching day per aviary. Indoor aviary groups consisted of an equal number of birds

(i.e. 7 or 8) per strain x prenatal treatment combination. The first week post-hatch food (Trutenküken Vormast, Kliba Nafag, Kaiseraugst, Switzerland) and water were provided *ad libitum*. From day 7 to day 29 post-hatch the postnatal feeding scheme was conducted, which consisted of food and water withdrawal during 3 hours a day at randomly varying time points between 8 am and 8 pm (in contrast to the parents held in outdoor aviaries, water withdrawal was feasible for the chicks in the indoor aviaries and adds to the unpredictability of resource availability; Levine & Ursin 1991). The birds that were allocated to postnatal predictable food supply received food and water *ad libitum* throughout the postnatal treatment phase. On day 29, the chicks were transferred into outdoor aviaries in Sempach (8 x 4 x 2 m) and were rearranged so that the new groups consisted of an equal number of birds of each strain x prenatal x postnatal food treatment combination (approximately four birds per combination) and of similar group size (approximately 32 birds per aviary group in total). Outdoor aviaries included hideaways of coniferous branches, sand bathing opportunities and natural grassy vegetation. Food and water were provided *ad libitum* all day. Throughout the study all aviaries were visited equally frequently.

Body mass at hatching was higher in domesticated strain chicks (mean hatching body mass domesticated = 10.1 g ± 0.09 SE; wild 9.4 g ± 0.07 SE; χ^2 = 12.14, d.f. = 1, P < 0.001), but did not differ between prenatal treatments (χ^2 = 1.85, d.f. = 1, P> 0.1). On day 55 after hatching (the day of blood sampling and vaccination, see below) body mass did not differ between strain x prenatal x postnatal food treatment combinations (χ^2 = 5.84, d.f. = 7, P = 0.55) but tended to be higher for males (mean body mass males = 234.3 g ± 2.5 SE; females 225.5 g ± 2.8 SE; χ^2 = 3.03, d.f. = 1, P = 0.08).

Sampling protocol

This section gives an overview of the sampling procedure. Methodological details are given in the following sections. In total we raised 1´360 chicks in 2010. A random subsample of 200 individuals (plus 48 control birds for the vaccination experiment; see below) from 13 aviary groups was used for this study (25 birds of each strain x prenatal x postnatal food treatment combination). On day 55 after hatching, these birds were blood sampled twice within 30 min by puncturing the brachial vein (about 100 µl blood). The first blood sample was taken to measure baseline level of corticosterone, the primary GC in birds. From the second sample we determined the GC stress response to an acute stressor, antibody baseline values

(measurement of adaptive immunity without prior immune challenge), innate humoral immunity, OSR and mSO production. On the same day these birds were injected with an immune stimulant to provoke a response of the adaptive immune system and an additional 48 with a control agent. Eight days after injection (day 63 after hatching) control and vaccinated birds were blood sampled again to measure antibody response (measurement of adaptive immunity after immune challenge). Blood samples were either analysed within eight hours (OSR, mSO production) or plasma was stored at -20° C until analysis (agglutination, antibody response and corticosterone). Sample size per parameter varied (see respective tables), because we could not obtain measurements of all indices for all individuals (in particular GC measurement was restricted to the 166 individuals blood-sampled within 3 min).

Determination of the baseline corticosterone and glucocorticoid stress response

Baseline and stress induced levels of corticosterone could be assessed in 166 birds. Baseline samples were obtained within 3 minutes after entering the aviary (first disturbance) and the GC stress response after 30 min of restraint in a cotton bag. Corticosterone level was determined using an enzyme immunoassay (Munro & Stabenfeldt 1984). In short, corticosterone was extracted from plasma using 4 ml dichlormethane and incubated overnight allowing adherence of corticosterone onto the well surface linked by the antibody (Chemicon; cross reactivity: 11-dehdrocorticosterone 0.35%, progesterone 0.004% 18-hydroxydeoxycorticosterone 0.01%, cortisol 0.12%, 18-hydroxycorticosterone 0.02% and aldosterone 0.06%). A horse radish peroxidase-corticosterone complex served as enzyme label and ABTS [2,2 –azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)] as substrate. Samples were evaluated in triplicates. Coefficients of variance were 2.5% and 22% within and between plates respectively. For a detailed description of the assay see Müller *et al.* (2007).

Determination of oxidative stress resistance

OSR was determined in 212 birds (200 individuals plus 12 from the PBS control group) using the KRL test (“SPIRAL” laboratories, Couternon, France) that was derived from human medicine and adapted to bird physiology (Alonso-Alvarez *et al.* 2004). The test measures OSR in a whole-blood sample, especially erythrocyte membrane resistance to oxidants which is tightly linked to vitamin E (Stocker *et al.* 2003; Monaghan *et al.* 2009). The test has

repeatedly been used to evaluate global antioxidant capacity including enzymatic and non-enzymatic antioxidants (Lesgards *et al.* 2002; Stocker *et al.* 2003; Bize *et al.* 2008).

180 µl of the KRL/blood solution (see above) was subjected to a controlled attack of ROS [2, 2'-azobis- (aminodinopropane) hydrochloride (AAPH)]. After initiating the ROS attack, the optical density (OD), i.e. consecutive cell densities in the sample, were repeatedly measured at 540 nm in 90 sec bouts at 37°C to determine the time needed to haemolyse 50% of the red blood cells in the sample. Initial OD, i.e. the initial density of cells in the sample, was assessed prior to the start of the assay and appeared to be an important predictor for haemolysis time.

Determination of adaptive humoral immunity

To determine an individual's ability to mount a specific immune response we conducted a vaccination assay. 200 birds were injected with 100 µl TETRAVAC vaccine (Sanofi Pasteur SA, Lyon, France), a polyvalent vaccine against diphtheria, tetanus, pertussis and poliomyelitis used for human vaccination and used to induce antibody production in birds (Stier *et al.* 2009; Gasparini *et al.* 2009). An additional 48 birds from the same 13 aviary groups representing all strain x prenatal x postnatal food treatment combinations were treated the same (handling procedures and feeding schemes) but received a 100 µl PBS (P3813, Sigma-Aldrich, Buchs, Switzerland) injection as control. Injected birds were recaptured 8 days after vaccination and a second blood sample was taken to determine the antibody response.

To determine antibody titre before and after TETRAVAC or PBS injection, we used a sandwich ELISA as described by Gasparini *et al.* (2009). Microtiter plates were coated with 100 µl of 1:50 diluted TETRAVAC vaccine for 2 h at room temperature. Plates were washed with PBS containing 0.05% tween (PBS-tween, tween 20, Reactolab, Servion, Switzerland) and unspecific binding sites were covered with PBS-tween containing 5% milk (PBS-milk, blotting grade blocker non-fat dry milk, Bio-Rad, Reinach, Switzerland) for 2 h at room temperature. After washing the plates they were mounted with 100 µl diluted plasma (1:80) and incubated over night at 4°C. The following morning plates were washed and equipped with 100 µl of peroxidase-conjugated rabbit anti-chicken IgG (A-9046, Sigma-Aldrich, Buchs, Switzerland) diluted in PBS-milk (1:3´000) and incubated for 2 h at room temperature. Plates were washed and 100 µl of peroxidase substrate (o-phenylenediamine dihydrochlorides, Sigma-Aldrich, Buchs, Switzerland) was added for 15 minutes at room temperature. Finally,

the reaction was stopped using 50 µl of hydrochloric acid (HCl 1M) and the optical density was measured at 490 nm wavelength using a photo-spectrometer (Wallac Victor 1420, Perkin Elmer LAS, Rodgau-Jügesheim, Germany). All samples were evaluated in triplicates and calibrated according to a standard consisting of four serial dilutions of a positive pool sample (1:50, 1:80, 1:100, 1:500). Repeatability within- and between plates was high (coefficients of variance 4.5% and 11.7%, respectively).

Determination of innate humoral immunity

To estimate an individual's potential to mount an unspecific, innate immune response we measured haemolysis and haemagglutination in 167 birds according to Matson, Ricklefs & Klasing (2005) with small modifications of the dilutions. Serial dilutions of plasma with PBS were prepared in 96 well round-bottom assay plates and 1% rabbit red blood cell suspension was added. Column one contained 20 µl pure plasma, whereas columns two to eleven were equipped with 20 µl of PBS. 20 µl of plasma was added to column two, mixed and 20 µl of the dilution was transferred to column three and so on. Hence, we tested serial dilutions of plasma ranging from 1 (pure plasma) to 1'024. Column 12 contained PBS only as a negative control. 20 µl of 1% rabbit red blood cell suspension (Harlan Labratories, An Venray, The Netherlands) was added to all wells. Plates were sealed, tilted and incubated at 37°C for 90 minutes. Plates were then photographed twice using a high resolution digital camera (Finepix S100, Fujifilm) and haemolysis and haemagglutination was scored according to Matson *et al.* (2005).

Determination of mitochondrial superoxide production

We measured mSO production for 210 birds (200 individuals plus 10 from the PBS control group) with the flow cytometric assay described by Mukhopadhyay *et al.* (2007) and Olsson *et al.* (2008). We used a molecular probe (MitoSox Red, M36008, URL http://www.invitrogen.com) that is oxidized specifically by superoxides but not by other ROS within the mitochondria. 16 µl of whole blood was diluted with 584 µl KRL buffer (150 mmol l^{-1} Na^{+}, 120 mmol l^{-1} Cl^{-}, 6 mmol l^{-1} K^{+}, 24 mmol l^{-1} HCO_3^{-}, 2 mmol l^{-1} Ca_2^{+}, 340 mosmol l^{-1}, pH 7.4) immediately after blood sampling. 404 µl of this solution was washed and dosed with 1 µl of 0.5 mM MitoSox. The rest of the solution was used in the analysis of OSR (see below). After an incubation period of 35 min (mean = 35.5 min ± 2.9 SD) at 37°C mSO generation was

measured using FACS Calibur flow cytometer and Cell Quest Pro software (BD Biosciences, URL http://www.bdbiosciences.com). Median fluorescence for a total of 50´000 cells per sample was determined and used for statistical analysis. Since time latency between incubation start and measurement can influence measurements and varied slightly between the samples, it was included as a covariate in the analysis.

Data analysis

We built mixed effect models with identity link function for each of the six following dependent variables: GC stress response (corticosterone levels after 30 min of restraint), adaptive immunity (antibody response to vaccination), innate immunity (haemagglutination and haemolysis scores), mSO production and OSR. The dependent variables were power transformed (Box & Cox 1964) if needed to suffice statistical assumptions. Prior to model selection all starting models included the following independent variables: Strain (wild, domesticated), pre- and postnatal feeding scheme as main effects and up to the three-way interaction; sex as main effect and in interaction with strain, pre- and postnatal feeding scheme (two-way interactions); body condition (residual body mass corrected for tarsus length); sampling date. Body condition and sampling date never proved to be significant, thus do not appear in the tables of the final models. Parental pair ID was included as random effect in all models. For the specific models we also included the following confounding variables: for the analysis of GC stress response we included GC baseline (fixed effect) and aviary group (random effect); in the analysis of OSR we included the initial OD (fixed effect) and lab series (random effect); in the analysis of adaptive immunity we included the type of vaccination (PBS control or TETRAVAC) as fixed effect and lab series (random effect); in the analysis of innate immunity we included lab series (random effect); in the analysis of mSO we included the latency between incubation start and measurement (fixed effect) and lab series (random effect).

To account for the interconnected nature of the physiological systems, we added in a second step the six other target variables (GC stress response, OSR, antibody response, agglutination score, lysis score, mSO) as main effects and in interaction with sex. Including predictor variables that might themselves be affected by the experimental treatment can conceal the potential effects of the treatment. Whenever there was a significant effect of

one of these additional independent terms we thus indicate whether and to what degree significance of the main terms (strain, pre- and postnatal food supply) changed.

All analyses were conducted using the generalized mixed effect procedure provided by the lme4 package (Bates, Maechler & Bolker 2012) in R 2.15 (R Development Core Team 2012). For the mixed model estimates P-values based on the χ^2-statistic were calculated using likelihood ratio tests (Bolker *et al.* 2009). We performed stepwise backwards model selection eliminating non-significant terms and performed likelihood ratio tests for each variable while leaving all other predictors in the model. We kept the main treatment predictors (strain, pre- and postnatal food supply) in the final models even if not significant.

For graphical representation and post-hoc comparisons we used a Bayesian approach and simulated joint posterior distributions from the final mixed models using the arm library in R (Gelman *et al.* 2012). We conducted post-hoc multiple comparisons of factors with more than two levels based on the simulated joint posterior distribution using a mixed model approach (Gelman, Hill & Yajima 2012). To do so the final models were refit and the factor with the levels to compare was added as random term. The shrinked group mean estimates were then used for multiple comparisons. From the joint posterior distribution of the model parameters, we derived the posterior distribution for each pair-wise difference between the means of the factor levels. These posterior distributions were used to obtain the probability of the hypotheses that the pair-wise differences were larger than zero P(Diff >0). We defined a post-hoc comparison to be significant if P(Diff>0) < 0.025 or > 0.975 in the case of a two tailed hypothesis, and P(Diff>0) < 0.05 or > 0.95 in the case of a one tailed hypothesis.

Results

Glucocorticoid stress response

The GC stress response was significantly affected by the interaction of strain x prenatal food availability and by corticosterone baseline values (Table 1). There was no effect of postnatal unpredictable food supply (Table 1). When testing for the effect of strain alone, it remained strong (χ^2 = 10.56, d.f. = 1, P = 0.001) with a higher response for wild than domesticated birds while there was no effect of prenatal unpredictable food supply alone (χ^2 = 0.52, d.f. = 1, P = 0.47).

Wild strain offspring subjected to pre- and postnatal predictable food supply had a higher GC stress response as compared to the corresponding domesticated birds (Figs 2A,

2C; posterior probability P(Diff >0) <0.001). Wild offspring subjected to prenatal unpredictable food supply had a lower GC stress response as compared to wild offspring in the prenatal predictable supply group (Fig. 2A, 2B; posterior probability P(Diff >0) = 0.02). There was no effect of the prenatal treatment within the domesticated group (Fig. 2C, 2D; posterior probability P(Diff >0) > 0.1), and there were no significant effects of OSR, innate or adaptive immunity or mSO production (all P-values > 0.1).

Oxidative stress resistance

Pre- and postnatal treatment affected OSR in the two strains differently resulting in a significant 3-way interaction (Table 1). The initial cell density was positively related to OSR (Table 1). However, initial cell density was not related to strain and pre- and postnatal treatments (all P-values > 0.2). Within all eight strain x prenatal x postnatal food treatment groups males showed higher OSR than females (estimate for males 66.38, SE range 64.77 to 67.98; females estimate 60.1, SE range 58.44 to 61.74, Table 1).

Wild strain offspring subjected to pre- and postnatal predictable food supply had a higher OSR than the corresponding domesticated birds (Figs 3A, 3C; posterior probability P(Diff >0) = 0.046). Within the wild strain, prenatal unpredictable food supply did not markedly affect OSR (Fig. 3A, 3B; all posterior probability comparisons P(Diff >0) > 0.1). OSR was drastically lower in wild birds encountering prenatal predictable and postnatal unpredictable food supply as compared to pre- and postnatal predictable supply (Figs 3A, 3B; posterior probability P(Diff >0) = 0.01), but OSR was not significantly affected when wild birds encountered both pre- and postnatal unpredictable food supply as compared to pre- and postnatal predictable supply (3A, 3B; posterior probability P(Diff >0) > 0.1). There was no effect of pre- and/or postnatal treatment within the domesticated strain (Fig. 3C, 3D; all pairwise comparisons within domesticated strain give posterior probabilities P(Diff >0) > 0.1), and there were no significant effects of GC stress response, innate or adaptive immunity or mSO production (all P-values > 0.1).

Adaptive immune system

Before stimulation of the adaptive immune system by TETRAVAC vaccination, there were no differences in specific antibody levels between control (PBS-injected) and immunized birds (χ^2 = 0.11, d.f. = 1, P = 0.74) and between strain x prenatal x postnatal food treatment

combinations (χ^2 = 3.32, d.f. = 7, P = 0.85), but females had higher initial baseline levels of antibodies than males (χ^2 = 4.35, d.f. = 1, P = 0.037). TETRAVAC vaccination successfully elevated antibody production within the same individual (before vaccination mean = 0.0037 ± 0.0042 SD; 8 days after vaccination mean = 0.0331 ± 0.0178 SD; pairwise t-test; t = -22.48, d.f. = 392, P < 0.001) and as a consequence antibody titre after vaccination differed greatly between vaccinated birds and PBS-injected controls (Table 2). Wild strain birds and birds subjected to postnatal unpredictable food supply showed a significantly higher antibody response after vaccination compared to the domesticated strain and the predictable supply groups (Fig. 4, Table 2). Antibody response decreased with increasing GC stress response in females but not in males (Fig. 5A, Table 2). When omitting GC stress response from the model effects of strain (χ^2 = 4.50, d.f. = 1, P = 0.034) and postnatal food supply remained significant (χ^2 = 5.16, d.f. = 1, P = 0.023), and females tended to have a higher antibody response than males (χ^2 = 3.18, d.f. = 1, P = 0.076). Prenatal unpredictable food supply did not contribute significantly to explain differences in adaptive immune response irrespective of whether GC stress response was included or not, and there were no significant effects of OSR, innate immunity or mSO production (all P-values > 0.1).

Innate immune system

Haemagglutination was significantly higher in birds subjected to postnatal unpredictable food supply compared to birds with a predictable supply (Fig. 4, Table 2). There was a significant interaction between strain and sex (Table 2). Domesticated males had lower haemagglutination score than domesticated females (estimate domesticated males 8.06 ± 0.19 SE and females 8.37 ± 0.22 SE) while the opposite was true for wild birds (estimate wild males 8.60 ± 0.2 SE and females 8.15 ± 0.21; Table 2). However, post-hoc tests did not reveal significant differences (all posterior probabilities >0.2). There were no significant effects of prenatal food supply, GC stress response, OSR, adaptive immunity and mSO production on haemagglutination (all P-values > 0.1).

Similar to haemagglutination birds subjected to postnatal unpredictable food supply had a higher haemolysis score than birds with a predictable postnatal food supply (estimate for predictable supply 1.98 ± 0.22 SE, estimate unpredictable supply 2.33 ± 0.22 SE; χ^2 = 4.86, d.f. = 1, P = 0.027) but no effects of strain, prenatal food supply or any interaction were

evident (all P-values > 0.1). In both analyses there were no effects of GC stress response, OSR, adaptive immunity or mSO (all P-values > 0.1).

Mitochondrial superoxide production

mSO production significantly increased with decreasing GC stress response (χ^2 = 3.93, d.f. = 1, P = 0.048, Fig. 5B), but there were no effects of OSR, immune measurements, sex and treatments (main terms and interactions) or any other covariates on mSO production (all P-values > 0.1). P-values for strain and treatment effects were all > 0.4 irrespective of whether GC stress response was included or omitted from the model.

Discussion

It appeared that both OSR and GC stress response were affected by strain and prenatal feeding scheme in a similar way. All measures of immune function were boosted by postnatal unpredictable food supply irrespective of strain and prenatal treatment. We also found that GC stress response values in plasma were negatively related to antibody response and mSO, which might highlight the regulative role of GCs in the context of resistance to oxidative stress (Fujita *et al.* 2009; Stier *et al.* 2009).

Effects of strain and food treatments on OSR and glucocorticoid stress response

Birds of the wild strain that were subjected to pre- and postnatal predictable food supply exhibited the highest GC stress response and, at the same time, they had the highest OSR of all groups. Notably, their GC stress response and OSR was markedly higher than that of their domesticated counterparts subjected to pre- and postnatal predictable food supply. This might reflect important consequences of the diverging selective pressures and adaptations under wild or captive conditions. The acute phase of the GC stress response is important to overcome life-threatening situations (Wingfield *et al.* 1998) and thus can be positively related to fitness (Breuner, Patterson & Hahn 2008). However, chronically increased GC levels adversely affect fitness traits (Cyr & Romero 2007) and are related to high levels of oxidative stress (Costantini *et al.* 2011). Likewise, high OSR has repeatedly been linked to enhanced fitness in birds from captive (Alonso-Alvarez *et al.* 2006; Kim *et al.* 2010b) and wild sources (Bize *et al.* 2008). We know of no other study comparing OSR of wild and domesticated conspecifics, but heritability of OSR has been found to differ between captive

and wild birds. While wild yellow-legged gulls (*Larus cachinnans*) showed a relatively high heritability of OSR at an age of 8 days ($h^2 = 0.59$) (Kim *et al.* 2010a), heritability of OSR in zebra finches (*Taeniopygia guttata*) kept and bred in captivity was low and non-significant at an age of 60 days ($h^2 = 0.05$) (Kim *et al.* 2010b). These results imply that there could be a reduced selective pressure on OSR in captivity. However, comparing heritability of OSR in different species living under different conditions can be ambiguous (Costantini & Verhulst 2009).

We found that the GC stress response of wild offspring was dampened when their parents were subjected to prenatal unpredictable food supply. This might at first sight suggest an adverse effect of the prenatal unpredictable food supply on the phenotype of wild offspring. However, the down-regulation of the stress response could also represent a predictive adaptive response (Breuner 2008; Love & Williams 2008a), which indicates that the offspring's physiology is being prepared to better match the expected future environment. The adaptive value of such a down-regulation of the GC stress response may include energy saving in an unpredictable or captive environment (Love & Williams 2008b). We argue that benefits of a down-regulated GC stress response could arise particularly in terms of decreased oxidative load. Indeed, wild birds subjected to postnatal unpredictable food supply but not prenatal unpredictable food supply (thus were not prepared for unpredictable postnatal food supply) showed a strong GC stress response but low OSR. In contrast, wild birds that did encounter prenatal unpredictable food supply (thus were prepared for unpredictable postnatal food supply) exhibited a low GC stress response and their OSR was insignificantly affected by postnatal unpredictable food supply.

In our case all birds were subjected to the same frequency of human disturbance, which could elicit a GC stress response. However, OSR of the highly stress-responsive wild birds was only reduced when a high GC stress response coincided with postnatal unpredictable food supply. Predictability is an important factor determining stressfulness of stimuli (Levine & Ursin 1991). Food unpredictability could enhance the impact of other stressors (Clinchy *et al.* 2004; Bauer *et al.* 2011) and/or impair a bird's ability to buffer corticosterone (i.e. reduce the biological activity of corticosterone) (Lynn, Breuner & Wingfield 2003). The combined effect of unpredictable food supply and frequent initiation of a GC stress response in captivity could pose an overwhelming oxidative pressure which would negatively affect OSR in unprepared birds. Consequently, a prenatal down-regulation

of the offspring's GC stress response could alleviate the oxidative threat. The situation could be different in a non-captive context were a strong GC stress response could efficiently help to overcome a real life threatening situation such as a predator attack (Wingfield *et al.* 1998).

Given the oxidative burden provoked by GCs (Stier *et al.* 2009; Costantini *et al.* 2011) it makes sense to physiologically couple a strong GC stress response to a strong OSR. Thus, pre- and postnatal permanent food supply enables a high OSR that is capable of absorbing a high GC induced oxidative load. Therefore these two traits might have coevolved through pleiotropic effects or symmorphosis (Ducrest, Keller & Roulin 2008; Cohen *et al.* 2012).

In the domesticated strain, no effect of pre- and postnatal treatment on OSR or GC stress response was noticeable. It seems that birds of the domesticated strain do not adapt their chicks to oncoming conditions, perhaps because the GC stress response is reduced anyhow as a result of domestication process (Evans *et al.* 2006) or they might have lost their ability for subtle fine-tuning (Price 1999). The domestication process leads to profound alterations of physiology and behaviour with detrimental consequences for survival in the wild (McDougall *et al.* 2006; Frankham 2008). Probably the most important change accompanying the domestication process is a reduction of sensitivity of animals to changes in their environment (Price 1999) which could also include the ability to affect offspring via parental effects.

Effects of postnatal treatment on immunity

In our study prenatal unpredictable food supply did not affect immunity but postnatal unpredictable food supply resulted in a stronger antibody response and a higher haemolysis and haemagglutination score five weeks after hatch in both strains and sexes. Thus, there appeared to be a lasting effect of postnatal unpredictable food supply early in life on aspects of immunity which could have important consequences for fitness later on.

Any form of malnutrition (too low or too high food-intake, low quality foods) can impair an adequate immune function (Perez de Heredia, Gomez-Martinez & Marcos 2012). In laboratory animals where health problems due to excessive food intake are frequent, moderate food restrictions were found to enhance immune function (Fernandes *et al.* 1997). Here, the positive effects on immunity are likely related to the maintenance of a healthy body condition (Perez de Heredia *et al.* 2012). Body condition in our case was not affected

by pre-and postnatal treatments and can therefore not explain the observed pattern in immunity. Similar to our findings, other experimental studies in birds revealed that low protein or low total nutritional supply in early life can have lasting enhancing effects on immune indices which were not solely explained by body condition but there appear to be further immune-enhancing effects of allegedly adverse early-life conditions (Gonzalez *et al.* 1999; Tschirren *et al.* 2009). The lasting boost of immune indices in grey partridges could reflect overcompensation for a reduced immunity during the treatment phase (Birkhead, Fletcher & Pellatt 1999; Tschirren *et al.* 2009).

Alternatively, postnatal food unpredictability could have altered the acute stress-induced distribution of immune cells in the body. The acute stress response seems to be important for supporting the redistribution of immune cells from source tissue over the blood stream and into target organs (Dhabhar *et al.* 2012). During the early acute stress response (up to 30 min) lymphocytes (immune cells which are part of the innate and adaptive system) are mobilized from reservoirs into the blood before they enter target tissues. GCs appear to facilitate this redistribution of immune cells out of the blood into the target tissue (Dhabhar *et al.* 2012). It is difficult to assess how an alteration of the redistribution patterns induced by postnatal unpredictable food supply could have affected an individual's immunocompetence in the field. However, considering acute stress induced redistribution of immune cells seems to be crucial when investigating immunity, particularly when dealing with highly stress-sensitive wild animals.

Correlations between physiological parameters

We found a negative correlation between the activity of the adaptive immune system and the GC stress-response in females but not in males. This suggests a potential trade-off between the adaptive immune system and the GC stress response. Effects of GCs on the immune system are well-known but appear to be complex and manifold (Sapolsky *et al.* 2000). In general, GCs at higher concentrations seems to dampen the activity of the immune system (Klein 2000; Sapolsky *et al.* 2000), but can differentially affect the innate and the adaptive system (Bourgeon & Raclot 2006; Stier *et al.* 2009). Similar to other studies conducted in non-captive species, we found a dampening effect of GCs on the adaptive immune system but no effect on the innate system (Bourgeon & Raclot 2006; Stier *et al.* 2009).

Lowering the innate immune system's activity (the first line of defence) in a stressful situation might be too risky, whereas reducing the adaptive system may be a more bearable risk, at least in the short-term. In an acute stress state high levels of GCs could act as a transient down-regulator of a highly active immune system which could save energy and reduce the risk of oxidative stress and immune overreaction (Sapolsky *et al.* 2000; Costantini & Møller 2009; Stier *et al.* 2009).

We did not find any negative relationship between the adaptive immune system and the GC stress response in males. Sex differences in immune activity have repeatedly been described in many taxa (Nunn *et al.* 2009) and in relation to the stress physiology (Merrill *et al.* 2012). Generally, females mount stronger adaptive immune responses than males (Nunn *et al.* 2009). The strong adaptive immune response provides females with a better protection and may ultimately increase their survival and longevity (Rolff 2002) but also increase the risk of autoimmune disease (Nunn *et al.* 2009) and oxidative stress (Costantini & Møller 2009). Indeed, in many species the incidence of autoimmune disease is higher in females than in males (Klein 2000). A negative feedback, in which high levels of GCs prevent a high antibody response and reduce the risk of autoimmune disease, might be especially valuable in females. On the other hand, further down-regulation of the already moderate antibody response in males might be too dangerous in terms of infection risk. Females had higher baseline values of antibodies than males and tended to mount a stronger antibody response to vaccination. The downside of this pronounced activity of the adaptive immune system might be reflected in a constantly higher oxidative pressure and thus a lower OSR in females than in males, as was found within all strain x prenatal x postnatal treatment combinations. Interestingly, other studies have also found sex-specific differences in OSR which could be due to sex-specific differences in immune system activity (Lesgards *et al.* 2002; Alonso-Alvarez *et al.* 2006; Bize *et al.* 2008). Additionally, in their meta-analysis Costantini *et al.* (2011) concluded that GCs induced a higher oxidative stress in females than in males.

We did not find any noticeable effect of the pre- and postnatal feeding schemes on the mSO production but the GC stress response was negatively related to the mSO generation irrespective of treatment, suggesting a second potential trade-off. mSO generation is tightly linked to metabolic rate, oxygen consumption and ATP production (Finkel & Holbrook 2000). Immediately after encountering a stressor the "fight and flight"

reaction is initiated (Bracha *et al.* 2004) which up-regulates the metabolic rate and increases oxygen consumption, ATP production and eventually mSO production (Balaban *et al.* 2005; Costantini *et al.* 2011). Hence, both mitochondrial activity and GC stress response represent considerable sources of ROS (Balaban *et al.* 2005; Costantini *et al.* 2011). Maximising both GC stress response and mitochondrial ATP production might cause an overwhelming oxidative pressure and put an organism at imminent risk of oxidative stress. Two negative feedback loops could theoretically have caused the negative correlation between GC stress response and mSO generation. First, GCs at high concentrations might suppress mSO production. Indeed, it was shown that corticosterone at high, but still within physiological relevant concentrations can inhibit mitochondrial ROS generation and ATP production (Fujita *et al.* 2009). Second, an increase of mSO production could act as a dampening modulating factor on the expression of a GC response to an acute stressor, similarly as prolonged flight (Jenni-Eiermann *et al.* 2009) or a reduced body condition (Kitaysky *et al.* 2005). Both mechanisms could aim to reduce a high cumulative oxidative load but entail the risk of an impaired GC stress response and reduced ATP production, respectively.

Conclusions and perspectives

This study investigated interactions between genetic setup (strains), maternal effects (prenatal food availability) and early-life conditions (postnatal food availability) on various physiological parameters of the immune system, GC stress response and oxidative balance in a non-model species.

Wild strain grey partridges held under predictable food supply conditions showed an array of superior physiological traits indicative of greater overall fitness in the wild. While the wild strain bird's physiological setup may not suit a captive environment, they appear to be able to adapt their offspring to captive conditions. Maternal effects could not be shown in the domesticated strain which could be due to the domestication process. Indeed, a loss of the ability to adaptively shape their offspring to the prospective environment in domesticated strain grey partridges could greatly diminish their fitness in the wild, thus reducing their suitability for re-introduction projects. We plan to further investigate the fitness of our experimental groups in the wild.

It appeared that indices of the immune system were positively affected by postnatal unpredictable food supply. Conducting moderate food restrictions in captivity may provide

an easy method to boost an animal's immune function in adulthood, although the method has to be evaluated de novo in each species. As we expect that an enhanced immunity positively affects fitness, this method of rearing may improve re- introduction projects using captive bred individuals.

Finally, we found negative correlations between circulating stress-induced GC levels, adaptive immune response and mSO production. Investigating physiological networks rather than single aspects of physiology will be crucial for understanding phenotypic expression and its impacts on fitness (Cohen *et al.* 2012). Our study supports the importance of considering oxidative balance as a key element in the regulation of physiological systems which could ultimately add to the evolution of life-history strategies.

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Table 1. Results of the mixed model analysis of GC stress response and OSR. The effective sample size for the analysis of OSR was n = 212 and for GC stress response n= 166. Parental pair ID was included as random effect both models. Aviary group or lab series was added as random effect in the analysis GC stress response and of OSR, respectively. pre. trmt= prenatal treatment, post. trmt= postnatal treatment, n.a= not applicable, n.s.= not significant and removed from the final model.

	Glucocorticoid Stress Response			**Oxidative Stress Resistance**		
Independent variables	χ^2	d.f.	P	χ^2	d.f.	P
Strain	16.09	1	**<0.001**	5.84	1	**0.016**
Prenatal treatment (pre. trmt)	1.56	1	0.21	0.73	1	0.39
Postnatal treatment (post. trmt)	0.23	1	0.63	2.24	1	0.13
Sex			n.s.	25.54	1	**<0.001**
Initial cell density			n.a.	12.17	1	**<0.001**
Glucocorticoid baseline	23.06	1	**<0.001**			n.s.
Strain * pre. trmt	5.81	1	**0.016**	2.45	1	0.12
Strain * post. trmt			n.s.	11.33	1	**0.001**
Pre. trmt * post. trmt			n.s.	0.88	1	0.348
Strain * pre. trmt * post. trmt			n.s.	5.84	1	**0.016**

Table 2. Results of the mixed model analysis of the adaptive and innate immunity. The effective sample size for the analysis of adaptive immunity was n= 171 and innate immunity n= 167. Parental pair ID and lab series were added as a random factor in both analyses. n.a = not applicable, n.s.= not significant and removed from the final model.

	Adaptive Immunity			**Innate Immunity**		
Independent variables	χ^2	d.f.	P	χ^2	d.f.	P
Strain	5.63	1	**0.018**	0.94	1	0.33
Prenatal treatment	0.32	1	0.57	0.00	1	0.99
Postnatal treatment	4.25	1	**0.039**	5.31	1	**0.021**
Sex	9.79	1	**0.002**	2.21	1	0.14
GC stress response	8.52	1	**0.004**			n.s.
PBS (placebo)	30.69	1	**<0.001**			n.a.
Strain x sex			n.s.	7.35	1	**0.007**
GC stress response x sex	7.28	1	**0.003**			n.s.

Fig. 1. Adult (foreground) and juvenile (around 3 weeks old, background) grey partridges in one of the outdoor aviaries in Sempach, Switzerland. Photograph by B. Homberger.

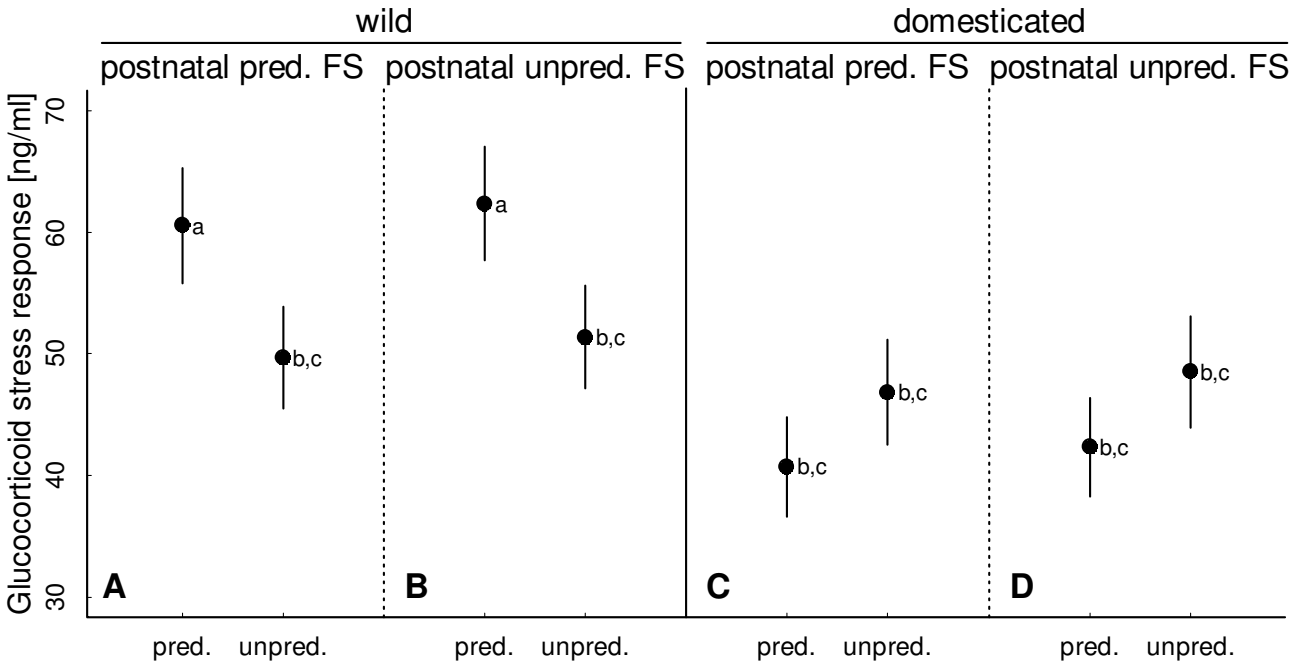

Fig. 2. Predicted means and standard errors for the two strains and the two pre- and postnatal food supply (FS) schemes on GC stress response. The x-axis indicates prenatal predictable (pred.) and prenatal unpredictable (unpred.) food supply. A: wild offspring, postnatal predictable food supply. B: wild offspring, postnatal unpredictable food supply. C: domesticated offspring, postnatal predictable food supply. D: domesticated offspring, postnatal unpredictable food supply. Predicted means are significantly different from each other ($p < 0.05$) when they do not share the same letters.

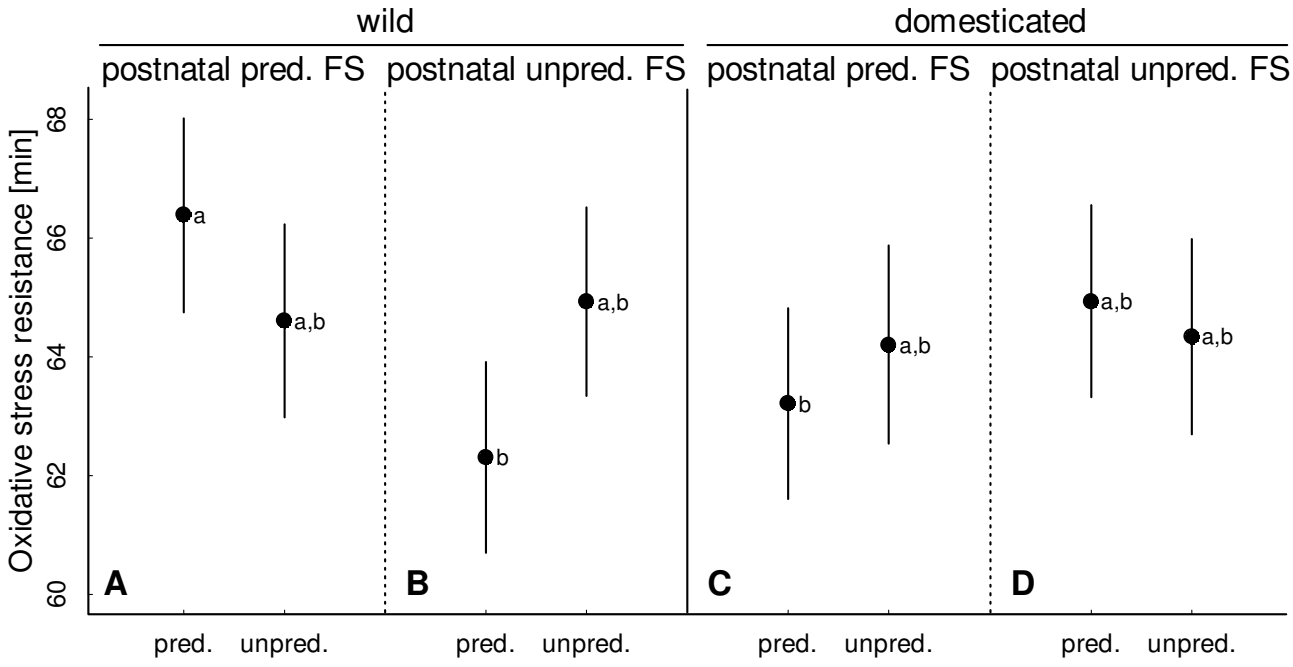

Fig. 3. Predicted means and standard errors for the two strains and the two pre- and postnatal food supply (FS) schemes on OSR. The x-axis indicates prenatal predictable (pred.) and prenatal unpredictable (unpred.) food supply. A: wild offspring, postnatal predictable food supply. B: wild offspring, postnatal unpredictable food supply. C: domesticated offspring, postnatal predictable food supply. D: domesticated offspring, postnatal unpredictable food supply. Predicted means are significantly different from each other ($p < 0.05$) when they do not share the same letters.

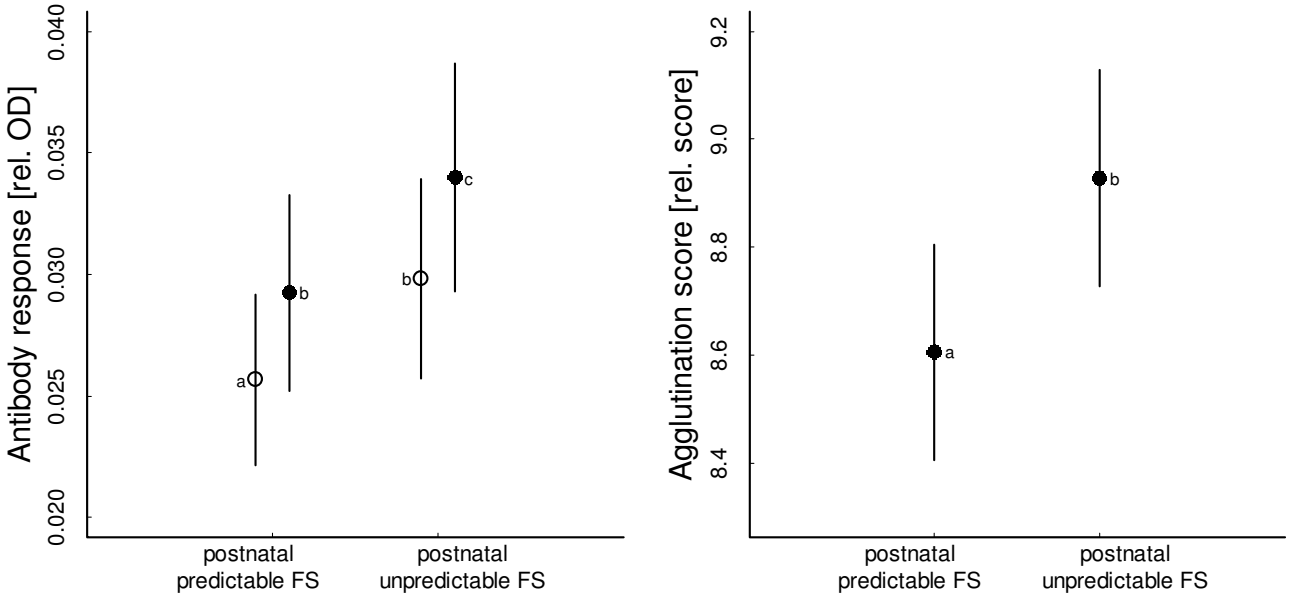

Fig. 4. Predicted means and standard errors for adaptive immunity (antibody response) on the left and innate immunity (agglutination score) on the right in relation to prenatal predictable and postnatal predictable and unpredictable food supply (FS). Open circles represent domesticated strain birds. Filled circles represent wild strain birds. Predicted means are significantly different from each other ($p < 0.05$) when they do not share the same letters.

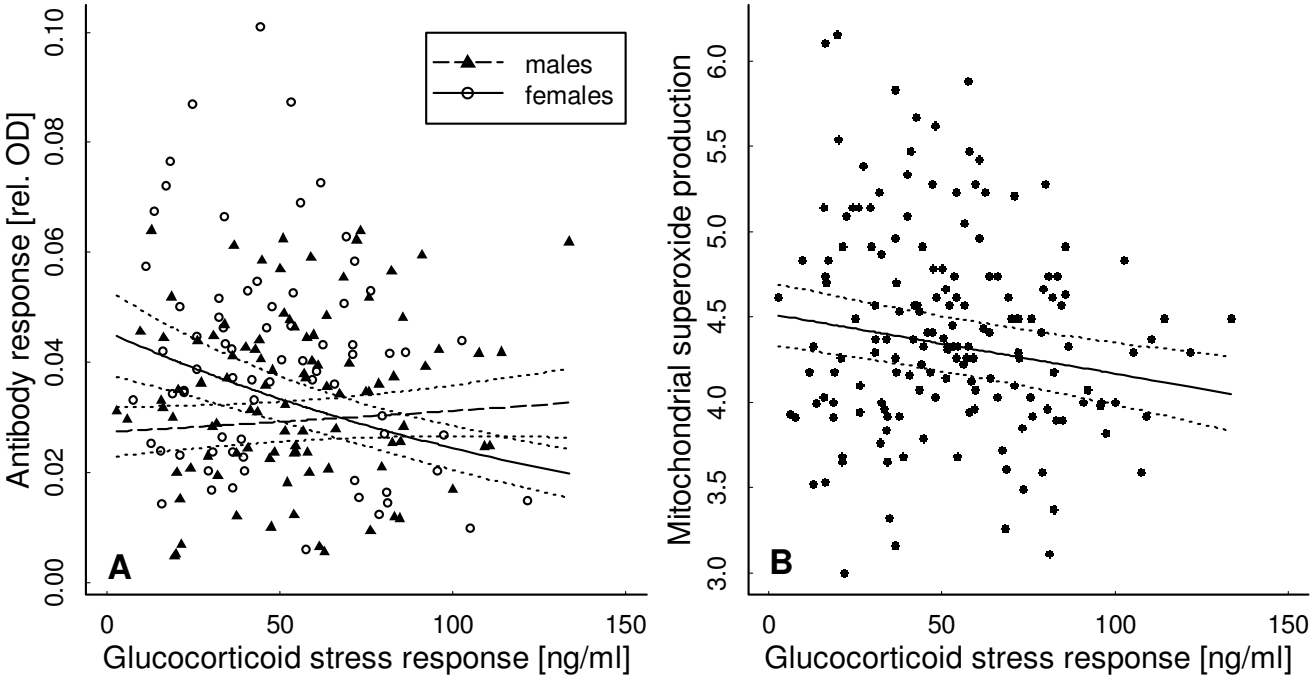

Fig. 5. Raw data points and model estimates with standard errors for the relationships between GC stress response and adaptive immunity (antibody production) (A) and mSO production (B). The panel on the left shows raw data and model estimates for males (triangles, dashed line) and females (open circles, solid line).

CHAPTER 2

Distinct responses of baseline and stress-induced corticosterone levels to genetic and environmental factors

Benjamin Homberger, Susanne Jenni-Eiermann & Lukas Jenni

Abstract

Glucocorticoid (GC) hormones, i.e. corticosterone (CORT) in birds, support physiological homeostasis and facilitate adaptations to stressful situations. However, high GC levels are energetically costly and interfere with other physiological processes. Keeping the balance of costs and benefits of GC hormones, various mechanisms act to adapt GC levels to environmental demands on different timescales, i.e. over generations, between parents and their offspring and within the life-time of a single individual.

We elucidated whether two strains (domesticated and wild) of grey partridges (*Perdix perdix*) differed in the developmental trajectories of baseline and stress response CORT throughout the first 80 days of life. We also explored the potential of prenatal and postnatal factors, i.e. parental origin, predictable vs. unpredictable food treatments, individual and social factors to modify these trajectories.

Baseline CORT was similar between strains and unaffected by perinatal food treatments. It decreased with age and was negatively related to body size and body condition. Conversely, the CORT stress response was hardly affected by physiological condition. It was stronger in wild than in domesticated birds and it increased with age. Within the domesticated strain, birds subjected to prenatal unpredictable food supply exhibited an accelerated development of the CORT stress response.

We conclude that the vital role of baseline CORT allows little adaptive scope since changes can quickly become detrimental. In contrast, the CORT stress response shows considerable adaptive potential which might ultimately support homeostasis in a changing environment.

Introduction

Glucocorticoid (GC) hormones secreted by the hypothalamic-pituitary-adrenal (HPA) axis are ubiquitous and crucial in vertebrate life (Sapolsky, Romero & Munck 2000). GCs orchestrate many physiological processes, including gene expression (Le *et al.* 2005), energy mobilization (Bonier *et al.* 2009), reproduction (Schmid *et al.* 2013), and they also interact with behaviour (Coppens, de Boer & Koolhaas 2010). The physiological role of GCs is quite distinct depending on whether they circulate at low baseline or at high acute stress induced levels. GC hormones at low baseline levels exhibit pronounced diurnal patterns and bind to mineralocorticoid receptors. They are involved in maintaining physiological homeostasis through allostatic change in everyday life (Romero 2004, Bonier 2008). In contrast, GC hormone levels rises drastically within minutes in response to external stressors and bind mainly to glucocorticoid receptors. The GC stress response adjusts physiology and behaviour to the demanding requirements of a stressful event and consequently helps to regain homoeostasis after the stressor has been overcome (Wingfield *et al.* 1998; Sapolsky *et al.* 2000).

The distinct patterns and physiological role of GCs at baseline and stress response level have been well described in many species, however, their fitness consequences and adaptive value for wild animals is less clear (Breuner, Patterson & Hahn 2008; Bonier *et al.* 2009). Thereby, important questions are whether GC excretion is flexible and what environmental factors can affect baseline and stress response during development. Flexibility of the HPA axis could be adaptive in that it integrates environmental cues and thereby help the individuals to efficiently cope with threads posed by a changing environment. It has been shown repeatedly that at least the GC stress response is to some degree under genetic control (Odeh, Cadd & Satterlee 2003; Almasi *et al.* 2010). However, the GC stress response is in competition for resources with other physiological processes and it implies costs and benefits which need to be traded-off (Wingfield & Sapolsky 2003; Costantini, Marasco & Møller 2011; Homberger *et al.* 2013). To adequately balance benefits and costs, the GC stress response is modulated by various intrinsic and extrinsic factors e.g. body energy stores (Schultner *et al.* 2013) or reproductive state (Schmid *et al.* 2013) and several mechanisms exist that could eventually shape an animal's GC stress response on different time scales. Apart from a relatively long term genetic adaptation, the GC stress response can be affected by maternal effects (Henriksen, Rettenbacher & Groothuis 2011)

and postnatal early-life conditions can induce transient or permanent alterations of the GC stress response (Lynn, Prince & Phillips 2010).

We aimed to investigate the relative contribution of prenatal and postnatal factors on the developmental trajectories of baseline and stress induced corticosterone (CORT) levels in a wild fowl species, the grey partridge. CORT is the primary GC of birds. We worked with two captive strains that varied in the duration they were kept and bred in captivity (thereafter called wild and domesticated strain). Parental pairs of both strains and their offspring were subjected to periods of predictable or unpredictable food supply. Unpredictable or insufficient food supply is a potent environmental perturbation which occurs frequently in the wild and could provoke physiological adaptations manifested in GCs levels (Bauer *et al.* 2011). Throughout the postnatal development we repeatedly measured levels of baseline and stress induced CORT levels and analysed whether genetic (strain and family origin), prenatal (food unpredictability, common prenatal environment) and postnatal (food unpredictability, social group, individual experience) factors can affect developmental trajectories of baseline and stress induced CORT levels.

We expected that wild strain birds exhibit a strong CORT stress response since this might help them to cope with the high predation threat they face in their natural habitats (Tapper, Potts & Brockless 1996). Conversely, in captivity an animal faces many intense but non-fatal stressors (e.g. handling, human proximity) and a high CORT stress response could be a disadvantage (e.g. through reducing reproduction) and thus selected against (Carrete *et al.* 2012). Therefore, we expected domesticated birds to have a reduced CORT stress response.

In birds, hormones deposited by the mother into the egg (e.g. testosterone and CORT), can affect the offspring's physiology, including the HPA axis (Sockman & Schwabl 2000; Henriksen *et al.* 2011). The prevalent hypothesis is that mothers can adapt their offspring to the prospective environmental conditions, predicted from the conditions prevailing during oviposition (Mousseau & Fox 1998; Love, McGowan & Sheriff 2013). We expected that prenatal unpredictable food supply encountered by the mother during oviposition would alter the development of the offsprings' CORT stress response as an adaptation to a potentially unpredictable postnatal environment. Postnatal insufficient or unpredictable food supply has been shown to affect baseline and stress response CORT levels (Kitaysky *et al.* 2001; Reneerkens, Piersma & Ramenofsky 2002). We hypothesized that postnatal

unpredictable food supply could induce an increase of baseline and stress response CORT levels. A high CORT stress response could facilitate feeding behaviour after a perturbation has been overcome and when food is available (Wingfield, Jacobs & Hillgarth 1997; Lohmus, Sundstrom & Moore 2006). However, a strong CORT stress response in stressful captive conditions could cause a chronic stress state manifested in increased baseline CORT (Kitaysky *et al.* 2001). In accordance with earlier findings we expected that the effects of pre- and postnatal feeding regimes were different in wild and domesticated birds, resulting in interacting effects (Homberger *et al.* 2013).

Finally, we examined the effects of additional intrinsic and extrinsic factors on developmental trajectories of CORT. We had repeated measurements of baseline and stress response CORT for multiple offspring from each parental pair, i.e. full siblings. This allowed us to test whether the full siblings resembled each other more than unrelated offspring in terms of the HPA function, and to estimate the effect of common parents in shaping variation in HPA axis functionality. Despite potential developmental constraints an individual might still be able to adopt its own stress coping strategies in accordance with its past experience (Wingfield & Sapolsky 2003). Thus, we estimated the variation between individuals and tested their contribution to the HPA axis function. To establish a coping strategy an individual has to acquire information from the environment (e.g. where are feeding grounds? Which predators are present?). Acquiring this information on an individual basis is risky. Alternatively, an individual could rely on public information provided by its social group, i.e. covey mates and even adjust the stress physiology accordingly. To test this we estimated the similarity of covey members in their stress physiological indices.

Materials and methods

Origin of birds

The grey partridge is a ground-dwelling game species with a wide distribution across Europe and western Asia. It is seasonally monogamous and typically has one clutch per year. After hatching, families form strong bonds which are maintained throughout the breeding season and the subsequent winter and only disintegrate during the consecutive spring (Potts 2012).

For our study we obtained grey partridge eggs from a UK breeder (Perdix Wildlife Solutions, Warwickshire UK) in 2009 and 2010. Eggs originated from two captive strains. For the first strain, male and female grey partridges were captured from a sustainable wild

population on a large wild game shooting estate in eastern England. Female offspring from these wild pairs were mated with males captured in the wild the consecutive spring. Offspring of this semi-wild strain were subjected to the prenatal treatment (see below) and produced part of the eggs for our study (subsequently called wild strain birds). Birds from the second strain were kept and bred in captivity for over 30 generations; no new wild-caught birds were added to this captive population (subsequently called domesticated strain). We assumed that the captive population of wild birds genetically closely resembles wild-living partridges, while in the captive strain adaptations to captivity might have occurred (Homberger *et al.* 2013).

Experimental procedure

During the breeding season, pairs of wild and domesticated partridges were held in outdoor aviaries (3 x 3 x 1.5 m) and randomly assigned to two feeding regimes. Pairs in the first feeding regime had 24 h access to food and water, hence access to food and water was fully predictable (consecutively called prenatal predictable food supply). In the second feeding regime food was removed during four hours a day at randomly differing times between 8 am and 8 pm in accordance with the UK regular feeding schemes. Thus, in the second feeding regime access to food was temporarily unpredictable (consecutively called prenatal unpredictable food supply). In 2009 we had a total of 22 parental pairs (11 of each strain) and subjected 5 pairs per strain to the predictable and 6 pairs per strain to the unpredictable feeding regime. In 2010, from a total of 50 parental pairs (25 of each strain), 12 pairs per strain were subjected to the predictable and 13 pairs to the unpredictable feeding regime. Eggs were collected daily, transported by airplane to Switzerland at weekly intervals and artificially incubated.

After hatching chicks were assigned to indoor aviaries (200 x 80 x 80 cm) with approximately 30 birds per hatching date and aviary. Indoor aviary groups consisted of an equal number of birds (i.e. 7 or 8) per strain x prenatal treatment combination. During the first week after hatching, birds received food (Trutenküken Vormast, Kliba Nafag, Kaiseraugst, Switzerland) and water *ad libitum*. From day 8 until day 43 in 2009 or until day 29 in 2010 half of the indoor aviary groups where subjected to an unpredictable food supply where food (and in 2010 also water) was withdrawn during 3 hours a day at randomly varying time points between 8 am and 8 pm (consecutively called postnatal unpredictable

food supply). The remaining half of the indoor aviary groups had a predictable 24 h access to food and water (consecutively called postnatal predictable food supply). On day 29, the chicks were transferred into outdoor aviaries (8 x 4 x 2 m). After the postnatal food treatment phase had ended birds were rearranged into outdoor aviary groups which were of similar age and size (approximately 32 birds per aviary group in total) and included an equal number of birds of each strain x prenatal x postnatal food treatment combination. From then on food and water were provided *ad libitum* for all birds. Outdoor aviaries resembled the natural habitat of the species with grassy vegetation, hideaways and sand bathing opportunities. Irrespective of the pre- and or postnatal feeding regime, all aviaries (indoors and outdoors) were visited by humans on a similar daily schedule for animal husbandry procedures.

Blood sampling and measurement protocols

In total 1016 chicks were used for blood sampling and measuring procedures. As many birds as possible were subjected to up to four blood sampling sessions throughout their development (for details see below) to determine plasma levels of CORT. At each blood sampling occasion we also obtained measurements of tarsus length and body mass. Each blood sampling session consisted of capturing the birds from the aviary and obtaining two blood samples (about 60 µl each) by puncturing the brachial vein with a needle and collecting the blood with a heparinized capillary tube (Brand GmbH, Wertheim, Germany). We took great care to get the first blood sample as fast as possible and the great majority of first samples (97%) were obtained within 3 minutes after first disturbance (Romero & Reed 2005). Time since first disturbance (thereafter handling time) was recorded separately for each blood sample. Samples obtained within a maximum of 250 sec after first disturbance were considered to represent baseline CORT levels. We also controlled for individual handling times in the analysis. After obtaining the first sample, birds were put in a cotton bag. A second blood sample representing CORT stress response levels was taken after 30 min since first disturbance. Blood samples were centrifuged within 2 hours after collection and plasma was stored at -20°C until lab analysis.

In both years, the first blood sampling was done when the chicks were 7 days old (prior to the postnatal food treatment phase) and the second blood sampling at 21 days (during the postnatal food treatment phase). The third blood sampling was conducted at an age of 45

days in 2009 (2 days after the postnatal treatment phase had ended), and 56 days in 2010 (17 days after the postnatal treatment phase had ended). We conducted a fourth blood sampling at the age of 80 days in 2009 only.

Corticosterone lab assay

CORT levels were analysed with an enzyme immunoassay (Munro & Stabenfeldt 1984). We used 4 ml of dichlormethane to extract CORT from plasma and incubated the samples overnight in presence of a CORT-antibody complex (Chemicon; cross reactivity: 11-dehdrocorticosterone 0.35%, progesterone 0.004% 18-hydroxydeoxycorticosterone 0.01%, cortisol 0.12%, 18-hydroxycorticosterone 0.02% and aldosterone 0.06%). As enzyme label we used a horse radish peroxidase-CORT complex and ABTS [2,2 –azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)] as substrate. The final CORT values we obtained from triple evaluations of each sample. Coefficients of variance were 2.5% and 16.1% within and between plates, respectively. For a detailed description of the assay, see Mueller *et al.* (2006).

Statistical analysis

Statistical analyses were performed using linear mixed effects models (Bates, Maechler & Bolker 2012) in the software R 2.12 (R Development Core Team 2012). We modelled developmental trajectories of baseline and stress response CORT. CORT values were transformed to obtain normally distributed residuals and all continuous variables were scaled by subtracting the respective overall mean from each value and dividing the outcome by the respective standard deviation. This allows direct comparisons of the effect sizes among variables (Gelman & Hill 2007). We conduced stepwise backward model selection by dropping non-significant terms (threshold P = 0.05) from an initial full model including all variables of interest (see below). We kept the main treatment predictors (strain, pre- and postnatal food supply) in the final models even if not significant. Significance of model estimates was derived from likelihood ratio tests based on χ^2-statistics comparing nested models fitted with maximum likelihood (Bolker *et al.* 2009; Zuur 2009). All graphs show 95% confidence intervals (CIs).

The full model of baseline CORT included the following predictors: Strain, prenatal treatment, postnatal treatment as main effects and up to four-way interactions of strain and

the two treatments with age, quadratic age and handling time. Age, quadratic age, handling time, current body condition (residual body mass corrected for age and tarsus length), current body size (residual tarsus length corrected for age), hatching body condition (residual hatching body mass corrected for tarsus length), sex and year were included as main effects. Finally, we included the two-way interactions of hatching condition with linear or quadratic age, respectively. The full model for the CORT stress response included the same terms, except for handling time. In both analyses we included individual ID (designating the individual bird), parent ID (designating the parents) and covey ID (designating the indoor or outdoor social group) as random effects. We calculated the intra-class correlation coefficients (ICCs) for each random effect which indicates the proportion of the total variance explained by each random factor. ICCs can be used to indicate the amount of within factor correlation or repeatability (Nakagawa & Schielzeth 2010).

Results

Baseline corticosterone levels

In both strains baseline CORT levels increased significantly within the 250 sec after capture but the increase was steeper in wild strain birds compared to domesticated strain birds (Fig. 1A, Table 1).

Raw mean values of the developmental trajectory of baseline CORT for the 8 strain x prenatal x postnatal treatment groups are given in Fig. 2. Baseline CORT decreased with age (predicted mean baseline CORT and CI for age 22 days = 6.37 (5.60 – 7.25) ng/ml and for age 57 days 5.29 (4.59 – 6.10) ng/ml; Table 1). Baseline CORT was lower in 2010 than in 2009 (predicted mean baseline CORT and CI for 2009 = 8.98 (7.57 – 10.66) ng/ml and for 2010 = 6.19 (5.45 – 7.04) ng/ml; Table 1).

Both, current body condition and current size were negatively related to baseline CORT but the effect of current body condition was stronger than the effect of current size (Fig. 1B, Table 1). There were no significant effects of strain, pre- and postnatal treatments on baseline CORT irrespective of whether current body condition and current size were included (Table 1) or omitted from the final model.

The ICCs of the random effects were < 0.01 for individuals (χ^2 = 0.01, d.f. = 1, P = 0.99), 0.03 for parents (χ^2 = 15.42, d.f. = 1, P < 0.001) and 0.13 for coveys (χ^2 = 71.18, d.f. = 1, P < 0.001). Hence, siblings (measured by ICC of parents) and especially members of the same

covey resembled each other in baseline CORT levels. Fig. 3A shows the predicted main effect sizes of strain and food treatments in comparison to the random effect deviations for baseline CORT. The standard deviations of baseline CORT between offspring of the same parents and between coveys were much larger than that between individuals. The main effect sizes of strain and the treatments were all relatively small.

CORT stress response

The CORT stress response increased throughout the first 50 days of life, and peaked at an age of 50 to 80 days (Fig. 4, Table 1), as it is also evident from the raw data (Fig. 2). Compared to domesticated birds wild-strain birds had a stronger stress response from an early age on (Figs 4A and 4B, Table 1) and within the prenatal predictable feeding group (Fig. 4A) they showed an accelerated developmental trajectory of the CORT stress response (Table 1).

Within the prenatal unpredictable feeding group (4B) wild and domesticated strain birds showed a similar increase in CORT stress response over time and both groups showed a peak of the CORT stress response at an age of around 60 days (Fig. 4B, Table 1). Within the domesticated strain (black curves in Figs 4A and 4B) birds subjected to prenatal unpredictable food supply (Fig. 4B) showed a steeper increase and an earlier peak of the CORT stress response compared to conspecifics subjected to prenatal predictable food supply (Fig. 4A,Table 1). Within the wild strain (grey curves in Figs 4A and 4B) birds subjected to prenatal unpredictable food supply (Fig. 4B) initially tended to show a stronger CORT stress response than those held under prenatal predictable food supply (Fig. 4A). However, from 20 days onwards there were no more differences in regard to the prenatal food supply in wild strain birds (cf. grey curves in Figs 4A and 4B).

In contrast to CORT baseline, current body condition and current size did not significantly affect the CORT stress response (Table 1). There was no significant effect of the postnatal food treatment irrespective of whether current body size and current body condition were kept in the model or omitted.

The ICCs were 0.09 for individual (χ^2 = 12.45, d.f. = 1, P < 0.001), 0.13 for parents (χ^2 = 72.11, d.f. = 1, P < 0.001) and 0.09 for covey (χ^2 = 53.74, d.f. = 1, P < 0.001) indicating similar substantial effects of all three random effects. Fig. 3B shows predictions of the main effect sizes in comparison to the random effect deviations for CORT stress response. The standard

deviations of stress response CORT were similar among parents, individuals and coveys and in the same order of magnitude as the main effect of strain.

Discussion

Plasma levels of baseline corticosterone changed only little over the postnatal development from the first up to 80 days of age. Current body condition, relative size and covey rather than the genetic background, maternal and other environmental effects influenced baseline CORT levels. In contrast, the CORT stress response increased with age and was affected by genetic factors (strain and parents) and prenatal food unpredictability. It varied with social context, but was hardly influenced by body condition and relative size. Hence, the relative contribution of strain, prenatal maternal and early postnatal effects on the phenotypic expression of two facets of the HPA-axis differed markedly between baseline and stress response CORT levels.

Development of baseline levels

There was no drastic change of baseline CORT levels throughout the development from a few days after hatching until about 80 days of age. A significant decrease of baseline CORT with age were mainly noticeable early in life (between day 7 and 20) and again at an age of 80 days. From 40 days onwards there was a considerable increase of variation in baseline CORT (Fig. 2).

CORT baseline levels decreased with increasing body condition, a pattern found in many other studies (e.g. Kitaysky *et al.* 2001). Less pronounced, there was also an additional negative effect of relative body size on baseline CORT which implies that not only poor body condition but also hampered growth can be correlated with high baseline CORT. Strain, pre- and postnatal feeding regimes did not significantly affect body mass, body size or body condition and consequently baseline CORT.

Individuals exhibited no repeatability in their baseline levels throughout development, which underlines the notion of a low temporal repeatability of baseline CORT in birds (Rensel & Schoech 2011; Ouyang, Hau & Bonier 2011). Importantly, however, members of the same covey had similar baseline CORT levels. The common environment of the covey, e.g. social interactions or disturbances prior to sampling, could have contributed to the similarity of baseline CORT levels within coveys. The social environment is an important modulator of an

individual's behaviour (Webster & Ward 2011) and social interactions and dominance rank within the group can affect the HPA axis (Creel *et al.* 2013).

CORT at baseline concentration is essentially involved in maintaining homeostasis in everyday life. It changes predictably throughout the day and ensures the energetic supply of the organism under normal or moderately (but not acutely) stressful conditions (Romero 2004). Baseline CORT seems to adjust primary according to the transient physiological state and energetic demands of the everyday environment. Hence, it does show considerable short-term flexibility but the potential for adaptations through maternal effects or natural selection appears to be low.

Development of glucocorticoid stress response

The CORT stress response increased from a few days after hatching throughout maturation; it peaked at an age of 50 to 80 days (depending on strain and prenatal treatment; discussed below) and then remained stable until the end of the observation period at around 13 weeks. The progressive increase of the CORT stress response throughout early life has repeatedly been found in birds (Wada, Hahn & Breuner 2007; Lynn, Kern & Phillips 2013) and could be a sheer consequence of physiological maturation (e.g. liver development) (Wada *et al.* 2007). However, reaching the full capacity to physiologically respond to a stressor early in life could be crucial especially for precocial chicks that have to cope with environmental threats from their first day of life (Wada 2008).

There is likely a trade-off between the benefits of an early fully responsive stress axis (i.e. being able to mount a full CORT stress response) and the risk of inferences of high CORT levels with hatchling growth and tissue maturation (Sapolsky & Meaney 1986; Wada 2008). A robust CORT stress response is crucial to cope with environmental perturbations but high CORT levels also impose costs and risks on multiple levels. There are direct physiological costs of glucocorticoids in the form of a high energy expenditure (Cote *et al.* 2006) and oxidative stress (Costantini *et al.* 2011). Juveniles appear to be generally more susceptible to CORT induced oxidative stress as compared to adults (Costantini *et al.* 2011). High levels of glucocorticoids can hamper immunity (Sapolsky *et al.* 2000) and interfere with mitochondrial activity (Fujita *et al.* 2009). In adult birds, inducing a CORT stress response as well as experimentally increasing CORT levels for longer periods can lead to a reduction of parental

care and might even provoke nest and brood abandonment (e.g. Goutte *et al.* 2010; Ouyang *et al.* 2013 but see Ouyang, Quetting & Hau 2012).

The subtle balance between benefits and costs of high CORT levels suggests that the CORT stress response has to be finely adjusted to the current condition of the bird and the intensity of stressors. Adaptations and modulations of the CORT stress response can be realized on various levels. Indeed, we observed strain differences (suggesting differing selection pressure in the past) and effects of the prenatal environment. In addition, we found evidence for individual and covey "strategies", but hardly any effect of postnatal food predictability and the transient physiological state. Intuitively, the strong CORT stress response of wild birds makes sense since it could help to adequately cope with threats they face in a natural, non-captive environment. However, direct evidence for survival benefits of a strong CORT stress response depends on the environmental context (Romero & Wikelski 2001; Blas *et al.* 2007; Cabezas *et al.* 2007; Breuner *et al.* 2008). In an immediately life-threatening encounter such as a predator attack, quickly mounting a strong CORT stress response might help to overcome the perturbation and facilitates re-establishment of physiological homeostasis afterwards (Wingfield *et al.* 1998; Landys, Ramenofsky & Wingfield 2006). In contrast, the lower CORT stress response of domesticated birds throughout early development could be a consequence of their captive breeding over many generations (Cabezas *et al.* 2013). Domestication is typically accompanied by a reduction of the sensitivity to environmental stimuli and can negatively affect traits that are important in the wild (Price 1999; McDougall *et al.* 2006; Frankham 2008). At the same time, reducing the CORT stress response in captivity could be beneficial. A strong CORT stress response will not normally help to overcome threats occurring in captivity (e.g. high density of animals or forced proximity to humans) but bears the risk of high stress induced physiological costs and impaired reproduction (Carrete *et al.* 2012; Homberger *et al.* 2013).

Besides the effect of strain there appears to be a considerable additive genetic variance in the CORT stress response between families (indicated by the relatively strong ICC of parents). In the absence of any non-genetic sources of resemblance among offspring sharing the same parents (common environment or parental effects), the variance explained by parents provides us with an estimate of the broad-sense heritability (H^2) of the CORT stress response of 0.26. This would suggest that the CORT stress response has the potential to respond to selection. Interestingly, this estimate is similar to (narrow-sense) heritability

estimates (h^2) of 0.14 and 0.30 for low and high CORT response breeding lines of Japanese quail (Odeh *et al.* 2003). However, it should be noted that, as our study was not designed for this specific purpose, our estimate is likely to be an overestimate of the adaptive potential of CORT response due to various non-additive genetic sources of resemblance among full siblings, including genetic dominance effects, as well as common environment and parental effects.

Domesticated birds responded to prenatal unpredictable food supply by accelerating the development of their CORT response trajectory. Consequently, they reached peak CORT response levels earlier as compared to their prenatally untreated conspecifics. This could reflect an adaptive maternal effect (Breuner 2008). Mothers adapted to a predictable captive environment could translate the sudden occurrence of unpredictable food supply during oviposition to their offspring and consequently accelerate their offspring's CORT stress response development. Prenatally prepared offspring might explicitly benefit from an earlier, more robust CORT stress response in terms of a facilitated foraging behaviour, a likely auspicious strategy in an environment where food availability is unpredictable (Wingfield *et al.* 1997; Wingfield & Kitaysky 2002; Cote *et al.* 2006). Benefits could also include improved fleeing behaviour (Chin *et al.* 2009) or a higher vigilance (Koolhaas *et al.* 1999) which appears to be a behavioural trait favoured by female grey partridges when they chose their mates (Dahlgren 1990).

A significant amount of variation in the CORT stress response can be explained by the individual. An individual grey partridge seems to adopt its own stress coping strategy in terms of the CORT stress response. This individual strategy could be based on previous experience, i.e. the way how a bird perceives and appraises stressfulness of the environment, rather than an innate routine. Even more, an individual grey partridge seems to match the CORT stress response to its covey which could facilitate flocking behaviours. Cooperative vigilance and coordinated flushing are integral parts of grey partridge behaviour which could be supported by a synchronized HPA axis activity (Tillmann 2009a; Tillmann 2009b; Creel *et al.* 2013). Adjusting the stress response to the group fellows could lead to a more united and coordinated behaviour of the covey. An individual whose HPA axis functionality and associated behaviours strongly deviate from the group mean is more exposed and faces an increased mortality risk during a predator attack. Clearly, there are

profound but often neglected interactions between the social environment and the HPA axis (Creel *et al.* 2013).

Postnatal perturbations such as food scarcity or handling can profoundly affect the HPA axis of juvenile and adult birds (Lynn *et al.* 2010; Cohen *et al.* 2012; Lynn *et al.* 2013). In this study, we did not find any significant lasting effect of postnatal unpredictable feeding on the CORT stress response trajectory. However, during and shortly after the postnatal treatment phase (between 20 and 60 days after hatching) birds subjected to unpredictable feeding appeared to have a slightly lower CORT stress responses compared to birds having predictable access to food (Figs 2B and 2C), but this difference was not statistically significant.

Conclusions

We found distinct developmental trajectories of baseline and stress response CORT. Baseline CORT primarily responded to proximate factors, i.e. momentary energetic demands and availability of resources, but did hardly change throughout development. There seems to be no room for heritable, maternally determined or individually acquired variation in baseline CORT. Enduring shifts of baseline CORT levels could hamper essential metabolic functions and quickly entail devastating effects on physiology and behaviour.

In contrast, the CORT stress response levels clearly increased throughout development and appeared to respond to selection and maternal effects. Environmental conditions can change rapidly especially in human altered habitats. In order to readily cope with such changes it could be important to adequately balance benefits and costs of the CORT stress response hence to flexibly adjust it to the demanding environmental conditions. It will be very valuable to investigate how other species from different habitats solve this trade-off and maintain HPA axis function in a changing world.

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Table 1. χ^2-statistics and associated p-values for the mixed model analysis of baseline and CORT stress response. The analysis of baseline CORT included 1920 observations from 771 individuals in 48 coveys. The analysis of CORT stress response included 1869 observations of 765 individuals in 48 coveys. Individuals, parents and coveys were included as random intercepts in both models. Non-significant terms were excluded from the final model except for the three main treatment effects. n.a. not applicable.

	Corticosterone baseline			**Corticosterone stress response**		
Independent variables	χ^2	d.f.	P	χ^2	d.f.	P
Strain	0.65	1	0.42	9.71	1	**0.002**
Prenatal treatment	0.78	1	0.38	1.67	1	0.19
Postnatal treatment	0.35	1	0.56	0.32	1	0.57
Handling time	130.61	1	**<0.001**			n.a.
Age	18.31	1	**<0.001**	39.20	1	**<0.001**
Age^2			n.s.	8.62	1	**0.003**
Current condition[a]	36.40	1	**<0.001**	3.71	1	0.054
Current body size[a]	6.31	1	**0.012**	1.15	1	0.28
Year	13.98	1	**<0.001**			n.s.
Corticosterone baseline			n.a.	100.67	1	**<0.001**
Strain x handling time	7.131	1	**0.008**			n.a.
Strain x prenatal treatment			n.s.			n.s.
Strain x age			n.s.			n.s.
Strain x age^2			n.s.	8.44	1	**0.004**
Prenatal treatment x age			n.s.			n.s.
Prenatal treatment x age^2			n.s.	5.86	1	**0.015**
Strain x prenatal treatment x age			n.s.			n.s.
Strain x prenatal treatment x age^2			n.s.	4.19	1	**0.041**

[a] Variables did not reach significance in the analysis of CORT stress response and were omitted from the final model.

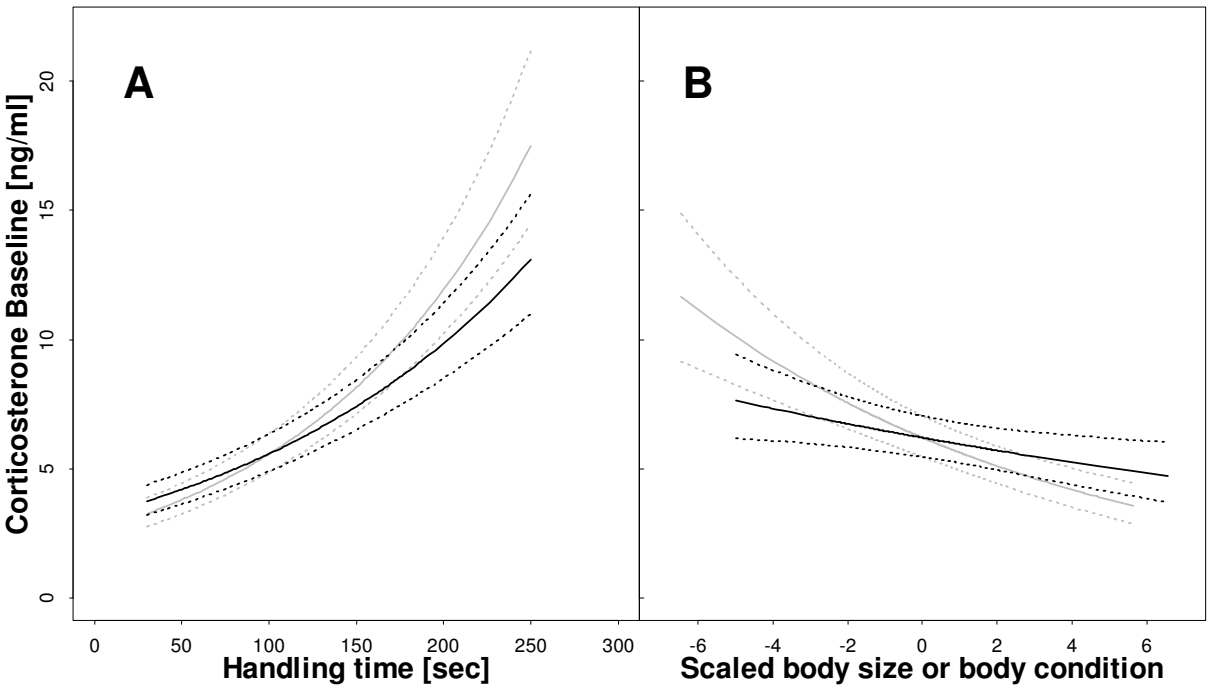

Fig. 1. Model estimates of baseline corticosterone analysis (Table 1). A) The increase of baseline corticosterone levels and CI for wild (grey line) and domesticated birds (black line) throughout the handling time (seconds since capture). B) Effects and CI of current condition (grey line) and current size (black line) on baseline corticosterone.

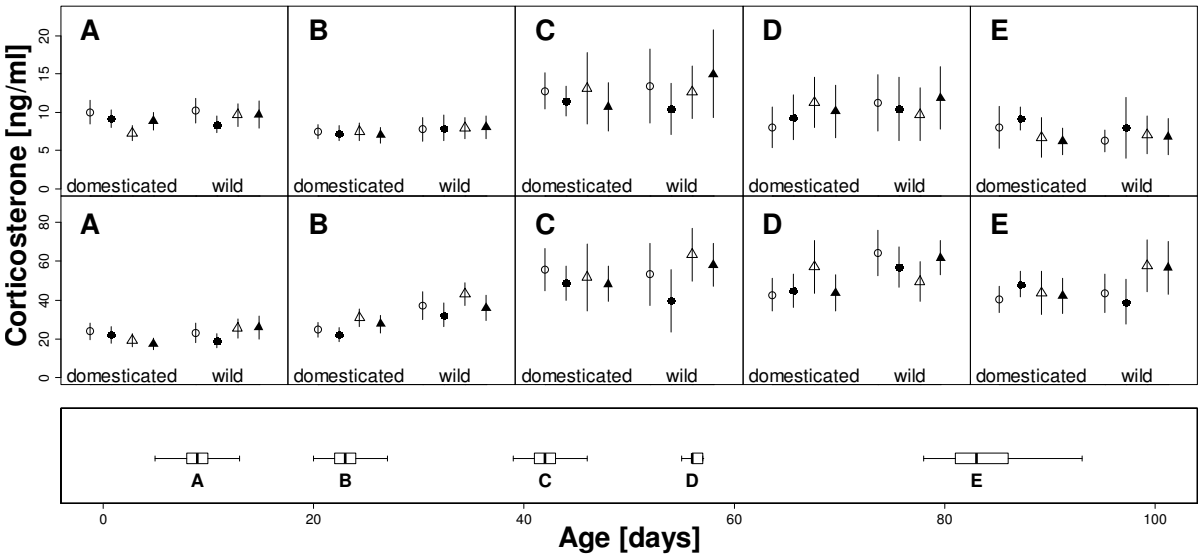

Fig. 2. Raw data mean values and CIs of baseline corticosterone (top row) and stress response (middle row) throughout development from chick to adulthood for the eight strain x prenatal x postnatal treatment combination groups. Strains are indicated in each panel. Within strains the pre x postnatal treatment combinations are ordered from left to right: pre- and postnatal predictable feeding (open circles), prenatal predictable and postnatal unpredictable feeding (closed circles), prenatal unpredictable and postnatal predictable feeding (open triangles), pre- and postnatal unpredictable feeding (closed triangles). The bottom row indicates boxplots of age ranges when samples were collected (capitals).

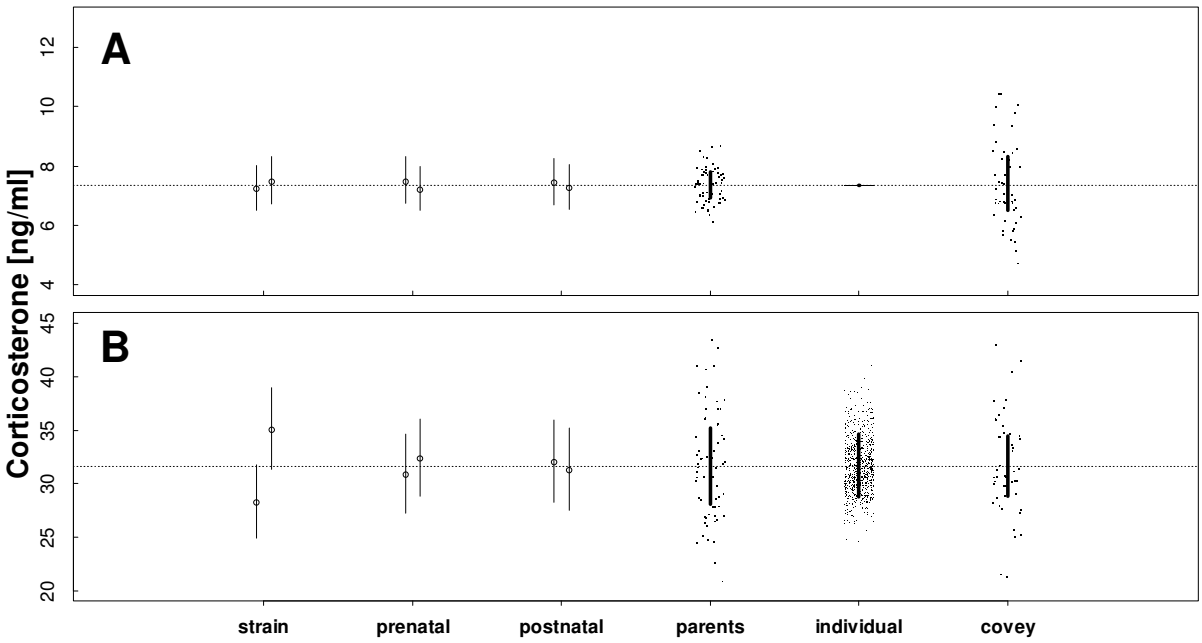

Fig. 3. Estimated main effects and CIs of strain (domesticated vs. wild), prenatal (predictable vs. unpredictable feeding) and postnatal treatment (predictable vs. unpredictable feeding) in relation to the random effects standard deviations derived from the analysis of corticosterone baseline (A) and stress response (B). Dots around the random effect standard deviations show best unbiased linear predictors for the respective random effects.

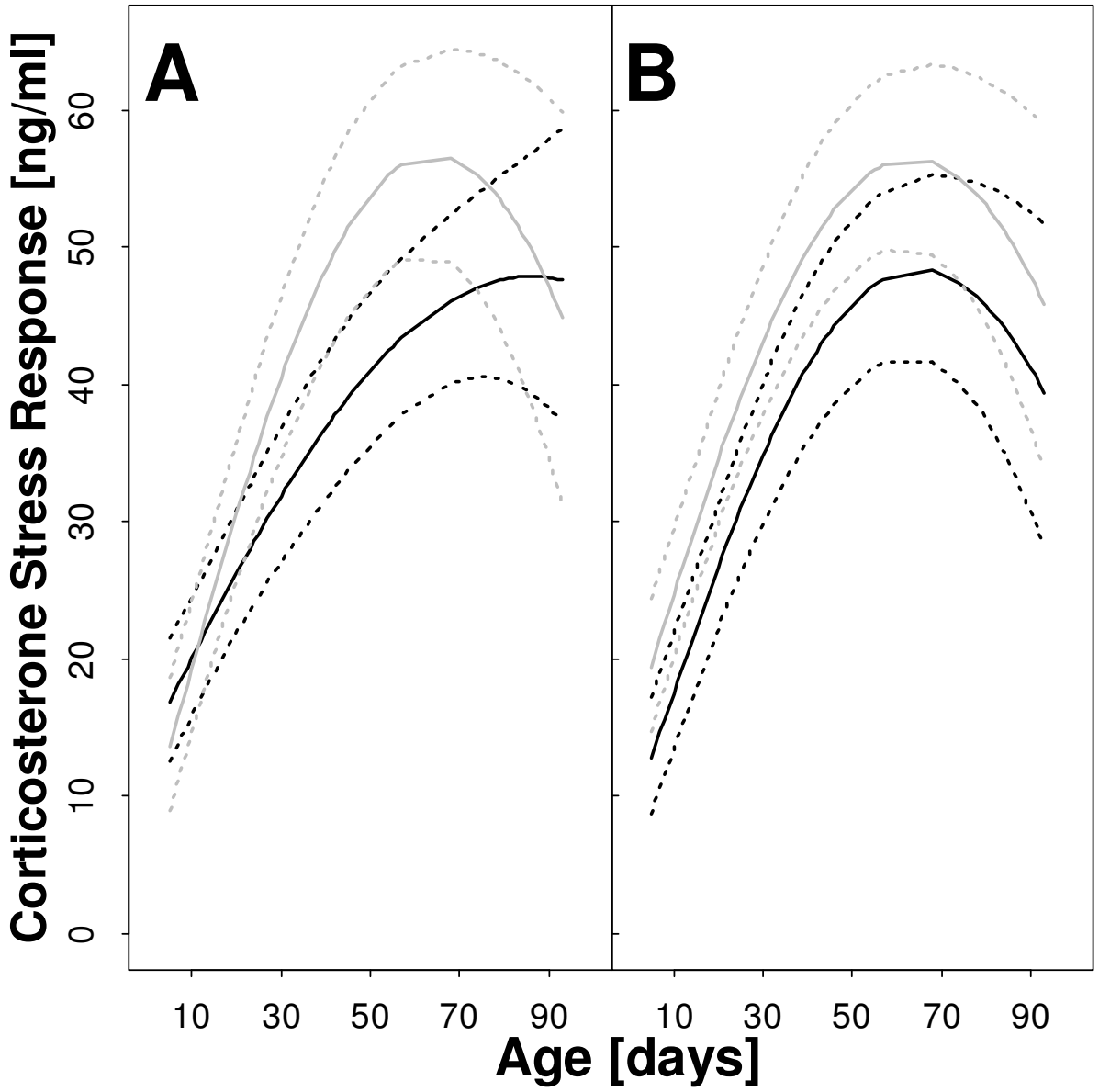

Fig. 4. The developmental trajectories and CIs of the CORT stress response over time for birds subjected to prenatal predictable (A) and prenatal unpredictable feeding (B). Grey lines represent wild strain birds. Black lines show domesticated strain birds. The dotted lines show the respective CIs.

CHAPTER 3

Be quick or be dead: proactive behaviour affects survival of reintroduced grey partridges

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Abstract

Animals need a diverse behavioural arsenal to survive in a changing environment. Behaviours are often repeatable within individuals and correlated among contexts but the adaptive value of behaviours and their correlations are context dependent. The 'constraint hypothesis' predicts that behaviours and their correlations are relatively fixed across contexts and emerge due to few, strong proximate factors, i.e. pleiotropic genes and hormones. Alternatively, the 'adaptive hypothesis' predicts that behavioural correlations are domain-specific adaptations. Hence, multiple intrinsic and extrinsic factors could govern behaviour and thereby provide behavioural variety for selection to act upon.

To test these hypotheses we manipulated prenatal and postnatal food supply in two captive strains (wild and domesticated) of grey partridges (*Perdix perdix*). We then investigated whether genetic background, prenatal and postnatal (physiological) factors affected behaviour as measured with three behavioural tests (timidity, tonic immobility, sociability). We also investigated whether the three behavioural traits were correlated and affected grey partridge survival after release.

High timidity and high tonic immobility were associated with high baseline levels of the stress hormone corticosterone. Parental factors (genes and the parental environment) affected timidity whereas tonic immobility and sociability appeared to be affected by acquired individual strategies and the immediate environment. The social group affected all behavioural traits. Correlations between behavioural traits were weak. Survival probability increased with decreasing timidity and was highest for intermediate levels of sociability.

In accordance with the 'adaptive hypothesis' multiple factors affected grey partridge behaviour and provided at least some individuals with the proactive and bold behavioural setup required to survive the harsh reintroduction conditions.

Introduction

Surviving in a complex and changing environment requires a broad range of behavioural strategies; so it intuitively would make sense that adapting behaviour immediately according to context would be optimal in the wild. However, wild animals often do not show full flexibility in their behaviour. Instead behavioural traits are often correlated over time and between different contexts, resulting in consistent behavioural differences between individuals, i.e. behavioural syndromes (Sih & Bell 2008).

The existence of behavioural syndromes can have profound implications at multiple levels: in individuals behavioural syndromes can result in constrained, non-optimal behaviour; in populations behavioural syndromes can, for example, affect the success of the colonisation of new areas and animal invasions (Sih, Bell & Johnson 2004; Chapple, Simmonds & Wong 2012; Dochtermann & Dingemanse 2013). However, given the implications and widespread existence of behavioural syndromes in many species, we know surprisingly little about how behavioural syndromes ultimately emerge and how they persist in the wild (Sih *et al.* 2004; Dingemanse & Wolf 2010; Adriaenssens & Johnsson 2013). Therefore, it is important to understand whether and to what degree behavioural syndromes are adaptive and in which contexts they affect fitness.

The 'constraint hypothesis' posits that behavioural syndromes are relatively fixed across various contexts and emerge due to single pivotal mediating factors such as pleiotropic genes or hormonal correlates (Ketterson & Nolan 1999; van Oers *et al.* 2005; Dochtermann & Dingemanse 2013). Indeed, behavioural traits are partly heritable and candidate genes for behavioural consistencies have been found (Thomson et al. 2011). The concept of coping styles posits that consistent behavioural and neuroendocrine characteristics are linked and result in distinct (proactive vs. reactive) activity types (Koolhaas et al. 1999). Proactive, offensive behavioural types exhibit relatively low levels of glucocorticoids (cortisol in mammals or corticosterone in birds) whereas levels of these hormones are relatively high in reactive, passive and shy individuals (Koolhaas *et al.* 1999; Stoewe *et al.* 2010; Carere, Caramaschi & Fawcett 2010). Importantly, whereas clearly distinct behavioural types repeatedly emerged in studies selecting for either extremes of the proactive-reactive spectrum (Overli, Winberg & Pottinger 2005; Stoewe *et al.* 2010), wild populations likely consist of more intermediate, or a whole range of, behavioural types (Dingemanse *et al.* 2004; Sih & Bell 2008).

Contrasting the 'constraint hypothesis', behavioural syndromes could be domain-specific adaptations, i.e. natural selection would favour different sets of behavioural correlations depending on context (Wilson 1998; Smith & Blumstein 2012). Following this 'adaptive hypothesis', behavioural syndromes would be governed by multiple independent factors which can flexibly adjust behaviour to the environmental context, thus breaking with the rule that behavioural syndromes would hold across contexts. Beyond genetic factors maternal effects and postnatal conditions could prove especially important under the 'adaptive hypothesis' since they might enable more immediate adaptations of behaviour to the prevailing conditions. Different environmental contexts could modulate behaviour and entail a variety of behavioural types which would ultimately be selected or eradicated throughout ontogeny resulting in domain-specific behavioural syndromes (Adriaenssens & Johnsson 2013).

Our aim was to investigate whether we find support for the constraint or the 'adaptive hypothesis'. As suggested by Sih et al. (2004) we integrated data on morphology, physiology and genetic background to explain behaviour and its ecological consequences in a non-model species, the grey partridge (*Perdix perdix*). First, we investigated whether behaviour was primarily affected by parents, i.e. parental genes and parental environment and proximate physiology or whether behaviour could be modulated by unpredictable changes in the perinatal environment and by a dynamic social environment. Second we investigated whether there were correlations among behaviours reflecting syndromes. Finally, we investigated whether the behavioural traits affected survival after releasing the birds into the wild.

We expected that under the 'constraint hypothesis' there should be only a few, potentially strong factors (e.g. parents or corticosterone levels) influencing behavioural traits, and behaviour should be relatively stable and inert to changes in the prenatal and postnatal environment. Likewise, there should be strong correlations among fear-related behaviours. In contrast, high plasticity and responsiveness to diverse environmental factors and weaker correlations among behavioural traits would instead support the 'adaptive hypothesis'. In a reintroduction context animals face a completely unknown environment and obtaining information can be costly and risky. Thus, an adaptive strategy might be ineffective but behavioural constraints could pay off as a form of default specialization or fixed behavioural strategy (Sih & Bell 2008). Conversely, strongly correlated behavioural

traits, as postulated for the 'constraint hypothesis', can be risky if selection acts contrastingly on correlated behavioural traits and viable behavioural types would simply not exist. We were interested to see how selection acted on correlated behaviours under the severe selection bottleneck occurring after release into the wild.

Materials and methods

Origin of birds

The grey partridge is a ground-dwelling wildfowl species which historically was abundant but declined drastically in the last decades in many European countries (Kuijper, Oosterveld & Wymenga 2009). In Switzerland it became virtually extinct, thus the Swiss Ornithological Institute started a re-introduction project (Jenny, Holzgang & Zbinden 2005) and therefore imported grey partridge eggs from a breeder in the UK (Perdix Wildlife Solutions, Warwickshire UK) in 2009 and 2010. Parents that produced the eggs originated from two strains. For the first strain male and female grey partridges were captured from a sustainable wild population in eastern England. The consecutive spring female offspring from these wild pairs were mated with males captured in the wild and offspring of this semi-wild strain produced eggs for our study. These birds were considered to be genetically close to the wild population (subsequently called wild). Parents of the second strain consisted of birds that were kept and bred in captivity for at least 30 generations without adding new birds (subsequently called domesticated), thus likely had adapted to captivity (Homberger et al. 2013). In 2009 we had a total of 22 parental pairs (11 of each strain) and in 2010 we had 50 parental pairs (25 of each strain). The two strains differed in physiological indices (immunology, physiological stress response, oxidative stress resistance) (Homberger et al. 2013) and there is evidence for genetical differences based on microsatellite data (own unpublished data).

Experimental procedure

During oviposition (April to May in both years) parents were either subjected to a predictable feeding scheme with *ad libitum* access to food (subsequently called prenatal predictable food supply) (5 pairs per strain in 2009 and 12 pairs per strain in 2010) or access to food was denied during an unpredictable four hour time window between 8 am and 8 pm each day (6 and 13 pairs per strain; subsequently called prenatal unpredictable food supply).

Eggs were collected daily, transported to Switzerland weekly and artificially incubated. On the hatching day chicks were individually marked and assigned to coveys, i.e. social groups of approximately 32 birds (7 to 8 birds per strain x prenatal treatment combination). Coveys were housed in indoor aviaries (200 x 80 x 80 cm) during the subsequent four weeks. During the first week after hatching all birds had *ad libitum* access to food and water. Starting from the second week, half of the indoor aviary groups were subjected to a postnatal unpredictable feeding scheme where in 2009 access to food only and in 2010 access to food and water was denied during three hours a day at an unpredictable three hour time window between 8 am and 8 pm (subsequently called postnatal unpredictable food supply). The second half of the indoor aviary groups had *ad libitum* access to food and water throughout (subsequently called postnatal predictable food supply). All chicks were fed on a standardized chicken feed high in protein (Trutenküken Vormast, Kliba-Nafag, Kaiseraugst, Switzerland).

At an age of 29 days birds were relocated into outdoor aviaries (8 x 4 x 2 m) that resembled the natural habitat of the species. When the postnatal treatment phase had ended (age 44 days in 2009, age 29 days in 2010) birds were assigned to new outdoor aviary coveys consisting of approximately 4 birds per strain x prenatal x postnatal treatment combination and they received food and water *ad libitum* thereafter.

Behavioural testing

We performed three behavioural tests at an age of 44 (2009) and 52 (2010) days to obtain a single measurement from birds of the eight strain x prenatal x postnatal combination groups. Additionally, we conducted the same three tests 6 times in a subset of 47 birds from 3 coveys between July and November 2009 at an age of 33 – 121 days (at least one week between test repeats).

The three behavioural tests were carried out consecutively between 0800 and 1400 in in the order presented below. During the tests birds had no visual contact to the experimenter or, except for the separation test, to conspecifics. Tests were carried out in a similar aviary as the birds were held, but equipped with sun and rain protection. Firstly, an emergence test was conducted to measure timidity (i.e. fearfulness in venturing into a new environment) in an isolated context (Erhard & Mendl 1999; Davis *et al.* 2008). One bird was put into a wooden box (80 x 40 x 20 cm) which was placed in the test aviary. After

acclimatization for 5 min, the front slide-door of the box was opened remotely and the latency it took the bird to fully emerge from the box was measured. If a bird remained in the box for 10 min we stopped the test and assigned an emergence time of 600 sec.

Secondly, we measured tonic immobility (Jones 1986; Davis *et al.* 2008), probably the most widely used test for general fearfulness applicable to many species (Jones 1986; Forkman *et al.* 2007). To induce tonic immobility, the bird was gently restrained in a supine position by covering its feet and sternum with one hand and the head with the other hand. After 10 sec the hands were withdrawn and the time measured it took the bird to recover from tonic immobility. Tonic immobility could be induced in virtually all birds. If a bird did not recover from TI within 10 minutes the test was stopped and the bird was assigned a time of 600 sec.

Finally, a separation test was conducted to measure sociability, i.e. the urge for social reinstatement (Faure & Mills 1998; Reale *et al.* 2007). Similar to the emergence test, one bird was put into a wooden box (80 x 40 x 20 cm) which was placed in the test aviary. After acclimatization for 5 min, the front slide-door of the box was opened remotely and the focal bird had direct visual contact to conspecifics which were placed in a compartment at the other end of the test aviary, 6 m away. We measured the time it took the bird to return to its conspecifics, i.e. approaching the compartment housing the conspecifics within 10 cm. Again, if the birds remained in the wooden box for 10 minutes the test was stopped and the bird assigned a time of 600 sec.

Blood sampling and determination of corticosterone levels

At an age of 45 (2009) or 56 days (2010) we took blood samples of a subsample of birds that had been also subjected to behavioural tests. Blood sampling consisted of randomly capturing birds from their home aviary and obtaining a first sample within 3 minutes after first disturbance. This first sample was used to determine baseline levels of corticosterone in the plasma (Romero & Reed 2005). A second blood sample was taken 30 minutes after first disturbance to measure stress-induced levels of corticosterone. After blood sampling the birds were measured (tarsus, sex and body mass) and returned to their home aviaries. Blood samples were chilled immediately after sampling and centrifuged within 2 h. Plasma samples were then stored at -20°C until laboratory analysis.

To measure corticosterone from plasma samples, an enzyme immunoassay was used (Munro & Stabenfeldt 1984). Corticosterone was extracted from plasma using 4 ml dichlormethane and incubated overnight in presence of an antibody (Chemicon; cross reactivity: 11-dehydrocorticosterone 0.35%, progesterone 0.004% 18-hydroxydeoxycorticosterone 0.01%, cortisol 0.12%, 18-hydroxycorticosterone 0.02% and aldosterone 0.06%). A HRP-corticosterone complex served as enzyme label and ABTS [2,2 – azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)] as substrate. Samples were evaluated in triplicates. Inter- and intra-assay coefficients of variance were 3.0% and 22.1% respectively. For a detailed description of the assay refer to Müller et al. (2006).

Radio tagging and release of birds

At an age of approximately 110 days a subsample of birds were equipped with 11 g necklace radio transmitters with mortality switch (RI-2BM; Holohil Systems Ltd., Carp, ON, Canada) in due consideration of the tagging Guidelines for the Re-introduction of Galliformes for Conservation Purposes (IUCN 2009). Releases took place one week after tagging between mid-September and mid-November in the Champagne genevoise region in the southwest of Switzerland (6°04'E, 46°15'N). This region was the last natural stronghold of the species in Switzerland and was subjected to habitat improvements prior to the releases (Jenny *et al.* 2005; Lanz, Michler & Duplain 2012). After release the state (alive or dead) of each radio-tagged individual was recorded at least once per week throughout the nine months observation period using Yagi antennas (Titley Electronics Ltd, Ballina, Australia) and digital receivers (R1000 of Communication specialist Inc, USA and SIKA of Biotrack, UK).

Data analysis

The results are based on two separate datasets. The first dataset included a single measurement of emergence (392 individuals), tonic immobility (389 individuals) and separation (341 individuals) from birds of 22 aviary coveys. A subsample of this dataset included measurements of corticosterone (288 individuals) and survival, i.e. days survived after release (211 individuals from 19 coveys). The second small dataset included the six repeated measurements of each behavioural test from 47 individuals. The repeated observations dataset was used to estimate the random effects for individual and test date (individual and date-specific consistency in behaviour) but not to analyse survival or strain

and treatment effects (see below). Survival and behaviour measurements were right censored (survival observation was confined to 9 months, behavioural tests lasted for a maximum of 600 sec; Fig. 1).

Measures of behavioural tests were treated as time-to-event data, i.e. time until the bird showed the respective behaviour and analysed using Cox mixed-effects models (Ripatti & Palmgren 2000; Therneau, Grambsch & Pankratz 2003) in the statistical software R (Therneau 2012). Cox mixed-effects models account for the right censoring in the dependent variables. The exponent of their fixed effects gives the hazard ratio (HR), i.e. the proportional change of an event probability. The exponent of the standard deviation of the random effects indicates the average variation of the HR for this random effect (Pankratz, de Andrade & Therneau 2005).

We defined three full models (one for each behavioural test) for the single measurement dataset. Full models included strain, prenatal and postnatal treatment (as main effects and up to the three-way interactions), corticosterone measures (baseline or handling-stress induced), sex, body mass, year, linear and quadratic test date as fixed effects, and parent (designating an individuals' parents) and covey ID (designating an individuals' social group) as random effects. For the repeated measurement dataset we defined three full models with sex, linear and quadratic test date as fixed factors and parent ID, covey ID, individual ID and test date as random factors. We performed model selection by comparing nested models with likelihood ratio tests and backward eliminating non-significant terms from the full model until only the main terms of strain and prenatal and postnatal treatments and significant terms remained. When calculating correlations between behavioural tests we accounted for data censoring by calculating Pearson correlation coefficients based on medians of the coveys.

Survival was analysed using all birds which had undergone all three behavioural tests and were equipped with radio transmitters (177 birds). To account for double censoring (measures of survival and behaviour were censored), we fitted Cox mixed-effects models (Clayton 1994) using MCMC sampling in WinBUGS (Lunn et al. 2000). The behavioural measurements where assumed to be exponentially distributed: $x_i \sim \text{Exponential}(\lambda)I(c_i+)$, where x_i is either the time measured until the behaviour was observed or a missing value (NA) if the behaviour did not occur within 600 sec. The function $I(c_i+)$ left-truncates the distribution at the value c_i, which is 0 for observed behaviours and 600 if the behaviour did

not occur. In this way, we assumed that the right censored observations were higher than 600 with a probability distribution that was estimated from the observed values and the proportion of censored measurements. We used weak priors and conducted 100'000 iterations with a burn-in of 2000. After thinning we ended up with 10'000 simulations which yielded Rhat values all below 1.1 (Brooks & Gelman 1998). The full survival model included all three behavioural measurements in linear and quadratic form, sex, body mass prior to release, year and release date as fixed effects and parent ID, covey ID and release date as random effects. We consecutively eliminated predictors that showed posterior means not different from zero as assessed by their 95% credible intervals (CRI).

Results

Factors affecting behaviour

Emergence test

Emergence occurred in 66% of the birds (see Fig. 1 for a histogram of emergence times). The probability to emerge decreased with increasing baseline corticosterone (Table 1, Fig. 2). There were no significant effects of strain and treatments or any other predictors on emergence times (all P-values > 0.1; Table 1). Parents (χ^2 = 17.18, d.f. = 1, P< 0.001) and coveys (χ^2 = 28.93, d.f. = 1, P< 0.001) had significant effects on the probability to emerge (Fig. 3) indicating resemblance of this emergence within siblings and covey mates.

In the repeated measurement dataset emergence occurred in 76% of birds. There was a significant quadratic effect of test date on the probability to emerge (χ^2 = 19.77; d.f. = 1; P< 0.001). Emergence times decreased from test repeats 1 to 4 but eventually increased during repeats 5 and 6. There was no significant effect of sex on emergence (χ^2 = 0.95, d.f. = 1, P = 0.33). As in the single measurement dataset there was a strong effect of parents (χ^2 = 13.81, d.f. = 1, P < 0.001; Fig. 3) while the effects of coveys, individuals and test dates were small (all P-values > 0.5, Fig. 3).

Tonic immobility

Recovery from tonic immobility occurred in 82% of the individuals (Fig. 1). The probability to recover was higher in 2010 than in 2009 and decreased throughout the seasons in both years (Table 1). The probability to recover from tonic immobility significantly decreased with

increasing baseline corticosterone (Table 1, Fig. 2). There was a significant effect of covey (χ^2 = 14.24, d.f. = 1, P< 0.001; Fig. 3). The parent effect was not significant (χ^2 = 1.58, d.f. = 1, P = 0.21, Fig. 3).

In the repeated measurement dataset recovery occurred in 83% of birds. The probability to recover increased linearly with test date (HR ± s.e.= 1.01 ± 0.003; χ^2= 3.52, P< 0.002).There were no significant effects of sex (χ^2 = 2.87; d.f. = 1, P = 0.09), covey (χ^2 = 0.01, d.f. = 1, P = 0.91; Fig. 3) and parents (χ^2 = 3.04, d.f. = 1, P = 0.081; Fig. 3) but a significant individual effect (χ^2 = 10.65, d.f. = 1, P = 0.001; Fig. 3).

Separation test

Returning to conspecifics occurred in 84% of the individuals (Fig. 1). Males exhibited a lower probability to return than females (Table 1) and the return probability was around 6 times higher in 2010 than in 2009 (Table 1). None of the other predictors explained a significant part of the variance in the data (all P-values > 0.1; Table 1). Return probability strongly depended on covey (χ^2 = 116.87, d.f. = 1, P< 0.001; Fig. 3) and there was a significant effect of parents (χ^2 = 7.871, d.f. = 1, P = 0.005; Fig. 3).

In the repeated measurement dataset 85% of individuals returned. Linear and quadratic test date and sex did not significantly explain variance in separation times (all P-values > 0.2). There were strong individual (χ^2 = 18.04, d.f. = 1, P<0.001; Fig. 3) and test date (χ^2 = 23.16, d.f. = 1, P< 0.001; Fig. 3) effects while effects of covey (χ^2 = 2.56, d.f. = 1 ,P = 0.11; Fig. 3) and parents (χ^2 = 0.01, d.f. = 1, P = 0.94; Fig. 3) were not significant.

Correlations between behavioural traits

Covey medians of tonic immobility were not significantly correlated with emergence (r ± s.e. = 0.21 ± 0.19, d.f. = 20, P = 0.36) or separation medians (r ± s.e. = 0.25 ± 0.22, d.f. = 17, P = 0.31). Covey medians of emergence were positively correlated with the corresponding separation times (r ± s.e. = 0.51 ± 0.17, d.f. = 17, P = 0.02). This positive correlation relied essentially on a relatively high proportion of birds (11.1%) showing both, emergence and separation times of 600 sec (Fig. 4C) and on 49.1% of all birds showing emergence and separation times both below 300 sec (Fig. 4A). Interestingly, a high proportion of all birds (20.5%) showed emergence times of 600 sec but separation times below 300 sec (Fig. 4B),

while the opposite (emergence times < 300 sec, separation times 600 sec) comprised only 2.9% of all birds.

Relationship between survival and behaviour

At the end of the nine month observation period 162 of the 177 released birds were dead (92%) whereas the state of the remaining 15 birds was alive or unknown (8%). Survival depended on emergence and on separation times, but not on tonic immobility. Mortality risk increased substantially with increasing time to emerge (estimated effect size and 95% credible interval (CRI) = 0.26, 0.08 - 0.44; Fig. 5) and there was a quadratic effect of separation time on survival (estimated effect size and 95% CRI = 0.28, 0.011 - 0.56). Birds with medium separation times had higher survival probabilities than birds that returned fast and birds that did not return (Fig. 5). Release date had a strong positive effect on mortality risk (estimated effect size and 95% CRI = 0.38, 0.09 - 0.65).Tonic immobility and all other fixed effects (sex, body mass prior to release, year) did not explain a considerable part of the variance and were thus excluded from the final model. The average variation of the mortality risk among coveys was 18%, 23% among families, i.e. offspring of the same parents, and 26% among release dates.

Discussion

We found that timidity (emergence) and tonic immobility were positively related to baseline corticosterone. Strain and non-social environmental conditions (prenatal and postnatal food unpredictability, test date) hardly affected the three behavioural traits but covey, i.e. social group was a significant factor in all three tests. Repeated measurements revealed that especially timidity (emergence) was affected by parents (genes and parental environment) whereas tonic immobility and sociability (separation) were consistent within individuals, i.e. tonic immobility and sociability (separation) were more affected by individual strategies and the immediate environment than by parents. Timidity (emergence) and sociability (separation) were weakly, but significantly, correlated, potentially reflecting a fear-related behavioural syndrome. Timidity (emergence) and sociability (separation) were also related to survival after release in an unknown environment, but selection acted divergently, favouring low timidity but medium sociability.

Effects on behaviours

Increasing levels of baseline corticosterone were related to increasing timidity, tonic immobility and hence an increasing probability to remain passive throughout these two tests. This agrees with the expectations of the concept of proactive-reactive coping styles (Koolhaas *et al.* 1999). However, it is unlikely that highly variable levels of circulating corticosterone directly determine the behavioural response (Romero & Reed 2008; Williams 2008; Sih & Bell 2008; Koolhaas *et al.* 2010). Rather the link between baseline corticosterone levels and the two behaviours could be mediated by physiological state. Relatively slowly changing state variables (e.g. condition, energy reserves, size) have been proposed to underlie behavioural types (McElreath & Strimling 2006) and do affect hormonal levels (e.g. Kitaysky *et al.* 2001). Circulating levels of baseline corticosterone are typically used to indicate general condition (Bonier *et al.* 2009). Indeed, in a larger dataset of our study which included the individuals used here, we found negative correlations between two relatively slowly changing state variables and corticosterone. Both, current body condition (body mass corrected for age and tarsus length) and current size (tarsus length corrected for age) were significantly negatively correlated to baseline corticosterone (see chapter 2). Although body mass (or body condition) did not significantly affect behavioural traits in this study, the probability to remain passive throughout the timidity and the tonic immobility tests was pronounced in birds with especially high baseline corticosterone levels. Thus, a poor physiological state, manifested in high levels of baseline corticosterone, could entail timid, shy and passive behaviour. Individuals in a poor state with high corticosterone might be stuck with playing it passive, timid and shy to make the best of a bad job (Sih *et al.* 2004).

In an earlier study we found differences in physiology between the strains and in their response to the perinatal treatments (Homberger *et al.* 2013). However, strain and treatments did not significantly affect behaviours. Perhaps there simply was no selective pressure to differentiate behaviour between the wild and the domesticated strain in the past, or the impact of our feeding treatments might have been too weak to induce noticeable effects on behaviour. The effects of strain and perinatal treatments could also have been concealed by a high variance in behavioural measurements due to the captive environment (Bell, Hankison & Laskowski 2009).

Consistent individual behaviour is a central aspect of behavioural syndromes (Sih & Bell 2008; Bell *et al.* 2009) and often arises due to genetic factors (Dochtermann &

Dingemanse 2013). We found evidence for behavioural consistency in 2 of the 3 behaviours. Individual strategies, rather than genes and the parental environment, affected tonic immobility and sociability which implies that grey partridges integrate information from the experienced environment and adjust their own behaviour accordingly. Conversely, timidity seems to be affected by parents which could constrain immediate flexibility of this behavioural trait.

The strong covey effects on all three behavioural traits in the single measurement dataset suggest an eminent role of the social group in determining individual behaviour (Watson, Aebischer & Cresswell 2007; Webster & Ward 2011). Conformity, i.e. synchronizing individual behaviour with the other group members, is probably important for many social animals (Efferson *et al.* 2008; Webster & Ward 2011). Well-coordinated behaviours among group mates such as coordinated fleeing in response to a predator attack are integral to grey partridge behaviour and likely convey survival benefits to the individual (Tillmann 2009).

In summary, grey partridge behaviour as measured with the three tests seems to be determined by a mix of inherited genetic and parental effects (timidity) but also by acquired individual strategies (tonic immobility and sociability) and the social (all three tests) and immediate non-social environment (test date). This mixture of factors influencing behaviour could provide a variety of behavioural types and ultimately support the 'adaptive hypothesis'. In the repeated measures dataset effects of coveys were apparently weaker but random effect estimates based on only three coveys can be vague and should not be used for inference.

Correlations of behaviour and survival

The three behavioural traits, as measured with three tests during the same day, were only weakly (positive correlation between emergence and separation) or not significantly correlated (tonic immobility with emergence or separation).

The positive correlation between timidity (emergence) and sociability (separation) might appear unexpected since quickly emerging into an unknown environment might be expected to reflect a rather bold behavioural type while quickly returning to the group mates should be characteristic of shy individuals. This implies that the same individual that seems to be bold in an isolated context (emergence) shows rather fearful behaviour in a social context (separation). The positive correlation and the functions of these two traits

might make more sense when considering the motivations, decisions and actions involved and their consequences (Wilson 1998).

First, an individual has to be able and willing to show activity, i.e. actively behave in any given context (activity vs. passivity). Passivity could indicate that an individual is overwhelmed and cannot readily cope with challenges such as unknown environments or captive test settings. Alternatively, passivity could result from a physiological inability, i.e. poor state as manifested in high baseline corticosterone. About 11% of all birds were highly timid and did not use the chance to return to the conspecifics. These passive and overwhelmed behavioural types entered into the correlation with maximum values of 600 sec and essentially provoked the positive correlation between timidity and separation.

Second, the underlying motivation might determine the behavioural activity if an individual is able and willing to be active. Emerging fast into the unknown is a proactive, boldness-motivated behaviour, whereas in a social context quickly returning to and finding shelter among the covey mates would represent a reactive, fear-motivated response, i.e. a reaction to the presence of conspecifics. Indeed, about 20% of all birds tended to remain passive in an isolated context but quickly sought shelter among the covey mates in a social context, hence they reacted to the presence of the conspecifics. On the other hand, timidity and sociability both rely on (locomotor) activity, hence proactive individuals also tended to quickly return to the conspecifics which suggests that both tests to some degree also reflect general activity. However, diverging selection pressures after release favouring activity in isolation, but not in a social context, suggests that the two traits are indeed functionally different.

Consequently, grey partridges exhibited three broad behavioural types which together represented over 80% of all birds (Fig. 4) and these types differed in post-release survival. There were ‘passive overwhelmed’ and ‘reactive shy’ behavioural types whose post-release survival was low. Then, there were ‘proactive bold’ behavioural types which had good post-release survival. The best surviving behavioural types showing low timidity and medium sociability could not have readily existed under a strong correlation of timidity and sociability. However, due to the high variability in the two traits and their rather weak correlation there existed a high diversity of behavioural types which is central to the ‘adaptive hypothesis’ and provided at least some individuals with an appropriate behavioural arsenal to survive even under harsh conditions and fierce natural selection.

Although tonic immobility has repeatedly been used to quantify fearfulness in birds (Jones 1986), it did not correlate with the other fear-related behaviours in our study. We can think of at least four explanations for the lack of correlations. Firstly, tonic immobility could per se not belong to the realm of fear-related behaviours but rather have arisen independently as an adaptive anti-predatory behaviour (Sih *et al.* 2004). Secondly, strong selective pressure in the past might have disentangled tonic immobility from the other traits (Sih *et al.* 2004). However, we did not find an effect of tonic immobility time on survival in our study. Thirdly, a correlation might not be apparent at this specific ontogenetic stage but might be present at a later life history stage (Sih & Bell 2008). Finally, as mentioned earlier, the captive test setting we used could have blurred potential correlations (Bell *et al.* 2009).

Proactive behaviour (low timidity) was related to high survival which supports findings of previous studies (Reale *et al.* 2000; Dingemanse *et al.* 2004; but see Bremner-Harrison, Prodohl & Elwood 2004; Smith & Blumstein 2008) and could be especially pronounced in the context of planned introductions or unintended animal invasions (Chapple *et al.* 2012). However, strong biases in survival towards proactive behavioural types at an early reintroduction stage could entail adverse knock-on effects (Sih *et al.* 2004). We found evidence for a maladaptive behavioural carry-over that could be amplified by a survival bias towards bold grey partridge males. It is known from mate choice experiments that females prefer males that show high vigilance behaviour during courtship (Dahlgren 1990). Likewise, we found that timidity, as measured in our test, was positively correlated with male vigilance during courtship (own unpublished data). Thus, highly timid males show high vigilance and are thus preferred by females. However, they also suffer high mortality after release. Such conflicts between natural and sexual selection can arise from behavioural constraints and could be especially adverse in populations having suffered severe selection bottlenecks early after release. For example in grey partridge, if only bold males survived the early reintroduction phase, females would have to put up with non-preferred bold males (probably also imprudent fathers) which could lower their reproduction success. Such conflicts can have far-reaching effects on ecology, evolution and conservation of species (McDougall *et al.* 2006; Sih & Bell 2008; Dochtermann & Dingemanse 2013).

Surprisingly, high sociability was related to low survival. Given the biological importance of the covey in this species and its effects on behaviour throughout this study we expected that high sociability should support the group structure and thus be beneficial for

individual survival. We suggest that the high sociability - low survival relationship could primarily arise due to the reintroduction context. After the relatively stable and predictable captive environment, the turmoil of release could disrupt established covey structures and expose highly social individuals to a greater mortality risk. Conversely, more independent (less-covey focused) individuals might fare better.

Conclusion

We draw two main conclusions from this study. Firstly, the three behavioural traits appear to be relatively flexible (responsive to multiple factors) and were only weakly correlated. Especially the social context and individual experience appear to be important modulators of behaviour in grey partridge. Individuals can acquire their own behavioural strategies, i.e. they can adjust behaviour according to context and ultimately exhibit adaptive individual differences in behaviour and domain-specific behavioural syndromes, a prerequisite of the 'adaptive hypothesis'.

Secondly, we found diverging selection pressures on behavioural traits, favouring behavioural types which would not readily be available under a strongly constrained behavioural syndrome. Strong constraints could seriously hamper the potential for appropriate behavioural responses whereas individuals which can show behavioural flexibility might experience a selective advantage especially in a rapidly changing environment.

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Table 1: Results of the final cox random-effects models for the analysis of three behavioural test measures as obtained from the single measurement dataset. Samples sizes were 321 for the emergence test, 320 for the tonic immobility test, and 338 for the separation test. Parent ID and covey ID were added as random effects in all analyses. The hazard ratio (HR) for continuous variables indicates the change of the occurrence probability per unit change in the explanatory variable. The HR for factors indicates the probability ratio that the respective behaviour occurred in an individual of the assigned level (e.g. wild vs. domesticated, prenatal predictable vs. prenatal unpredictable food supply etc.).

	Emergence				**Tonic immobilty**				**Separation**			
Independent variables	HR ± s.e.	d.f.	x^2	P-value	HR ± s.e.	d.f.	x^2	P-value	HR ± s.e.	d.f.	x^2	P-value
Strain (wild)	1.039 ± 0.21	1	0.03	0.87	0.867 ± 0.15	1	0.91	0.34	0.995 ± 0.16	1	0.00	0.97
Prenatal food supply (unpredictable)	1.012 ± 0.21	1	0.00	0.98	0.854 ± 0.15	1	1.12	0.29	0.830 ± 0.16	1	1.36	0.24
Postnatal food supply (unpredictable)	0.901 ± 0.15	1	0.42	0.51	0.939 ± 0.13	1	0.22	0.64	1.165 ± 0.13	1	1.30	0.26
Corticosterone baseline	0.977 ± 0.01	1	6.17	**0.013**	0.981 ± 0.01	1	3.89	**0.049**				n.s.
Corticosterone stress response*	1.002 ± 0.00	1	0.57	0.45	0.998 ± 0.00	1	0.20	0.65	1.004 ± 0.00	1	1.53	0.22
Sex (male)				n.s.				n.s.	0.733 ± 0.13	1	5.35	**0.021**
Body mass				n.s.				n.s.				n.s.
Year (2010)				n.s.	1.912 ± 0.23	1	6.82	**0.009**	6.592 ± 0.49	1	10.29	**<0.001**
Test date				n.s.	1.025 ± 0.01	1	13.33	**<0.001**				n.s.
Test date^2				n.s.				n.s.				n.s.
Strain x prenatal treatment				n.s.				n.s.				n.s.
Strain x postnatal treatmetn				n.s.				n.s.				n.s.
Prenatal x postnatal treatment				n.s.				n.s.				n.s.
Strain x prenatal x postnatal treatment				n.s.				n.s.				n.s.

*****coefficients and statistics for corticosterone stress response when included as a predictor instead of baseline corticosterone.

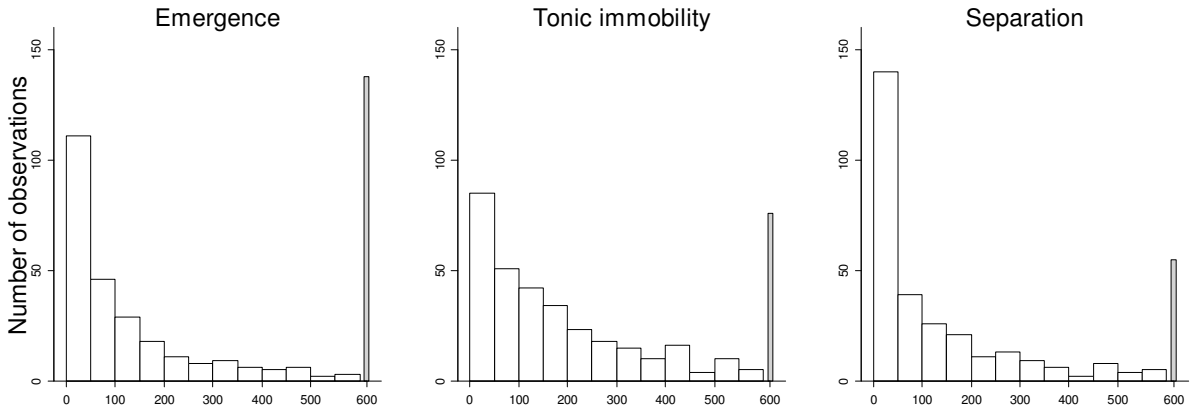

Fig. 1. Frequency distributions of the times to emerge (left), to recover from tonic immobility (middle) and to remain separated from conspecifics (right). The grey line at time 600 gives the number of individuals that did not show the respective behaviour within 10 min.

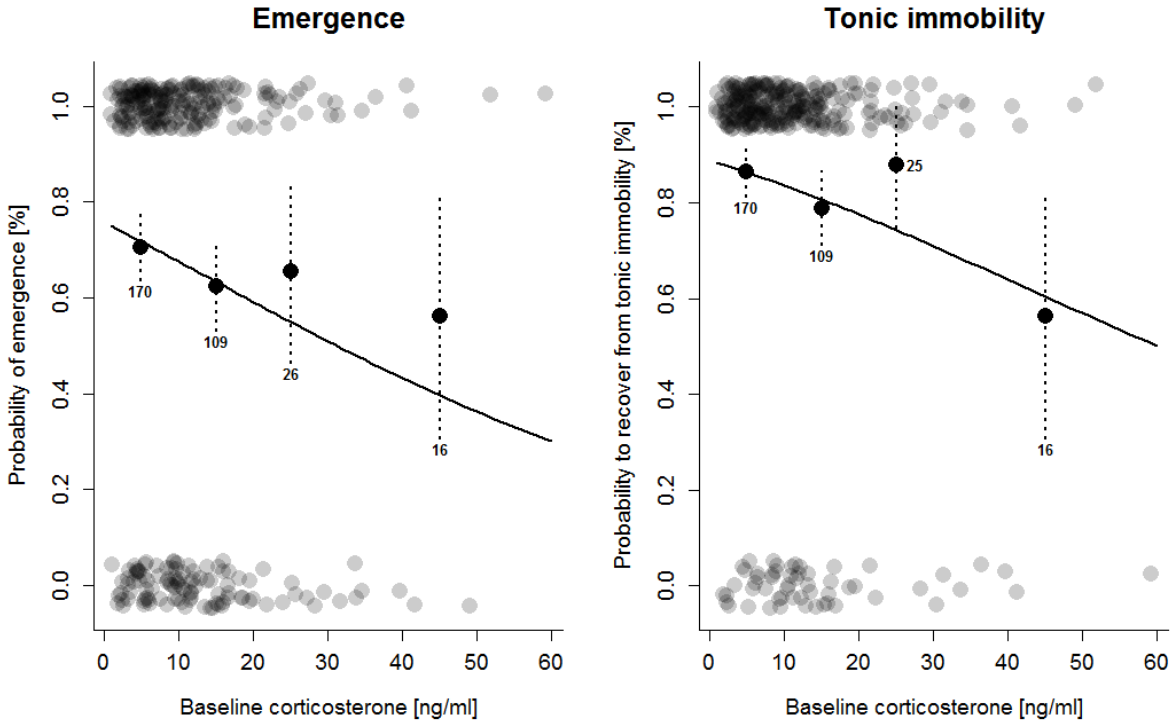

Fig. 2. Relationship between baseline corticosterone and the probability to emerge (left) or the probability to recover from tonic immobility (right). Lines indicate the predicted relationship from the final models of emergence and tonic immobility (see Table 1). Semi-transparent dots are the actual individual measures (two categories: at 1 = behaviour occurred, at 0 = behaviour did not occur). Dots with CIs and sample sizes are mean occurrence probabilities for four ranges (0 - 10, 11 - 20, 21 – 30, 31 – 60 ng/ml) of raw corticosterone values.

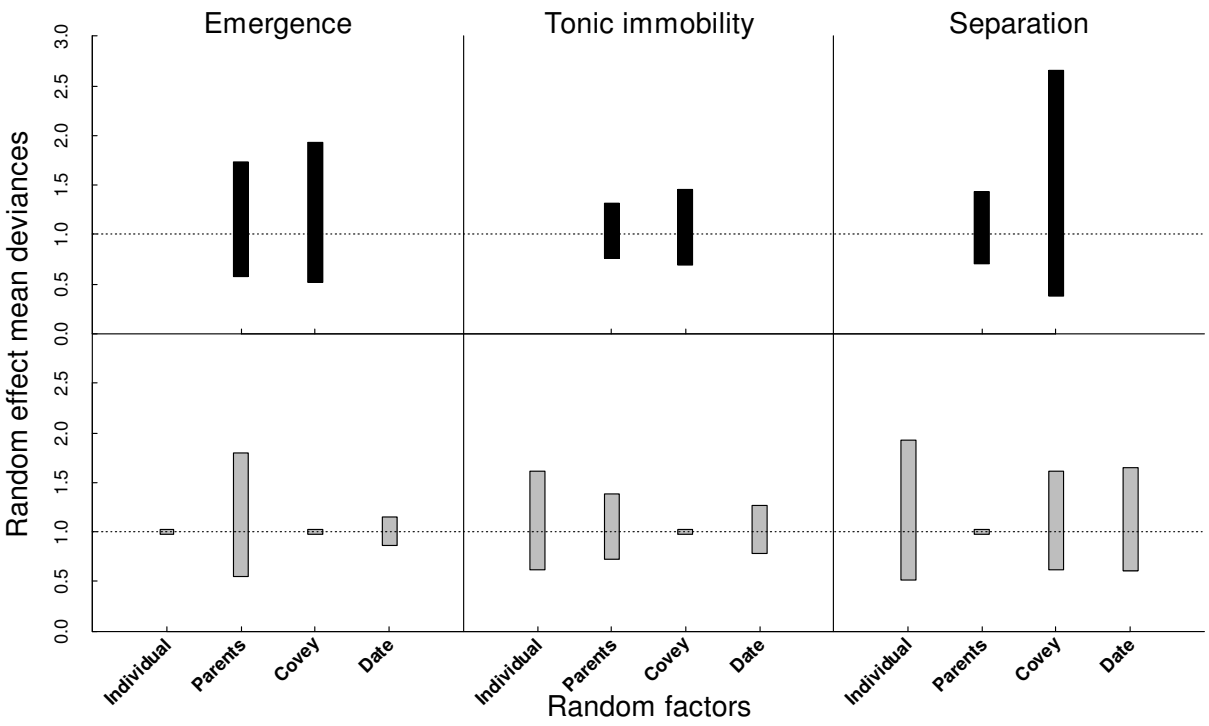

Fig. 3. Effects of the random factors on the three behavioural traits in the single measures dataset (upper three panels) and in the repeated measures dataset (lower three panels). The vertical bars indicate the average variation of the event probability for each random factor around an event probability of one (dotted lines at y=1). For example, the average variation in the emergence probability among families, i.e. the probability that offspring of the same parents emerged from the box within the 10 min test time ranged from around 0.5 to over 1.7 around a probability of one (bottom left panel).

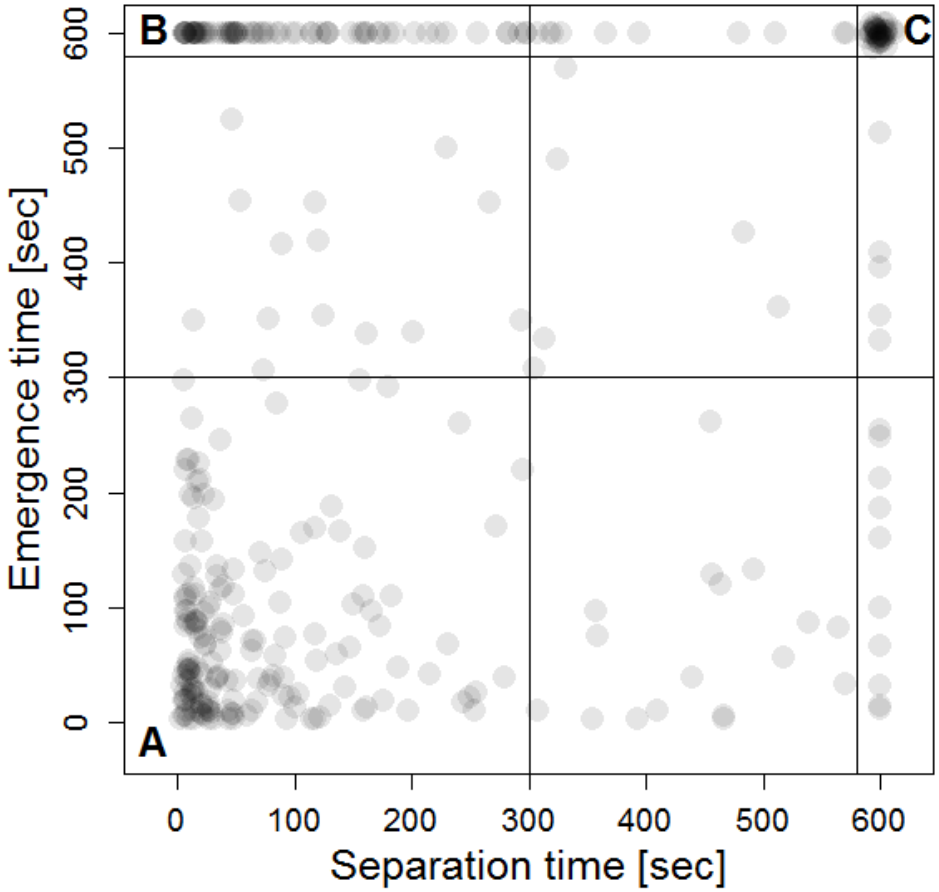

Fig. 4. Individual values of emergence vs. separation time (N=340) depicting the 3 broad behavioural types: A) 'proactive bold' (49.1%); B) 'reactive shy' (20.5%); C) 'passive overwhelmed' (12.0%).

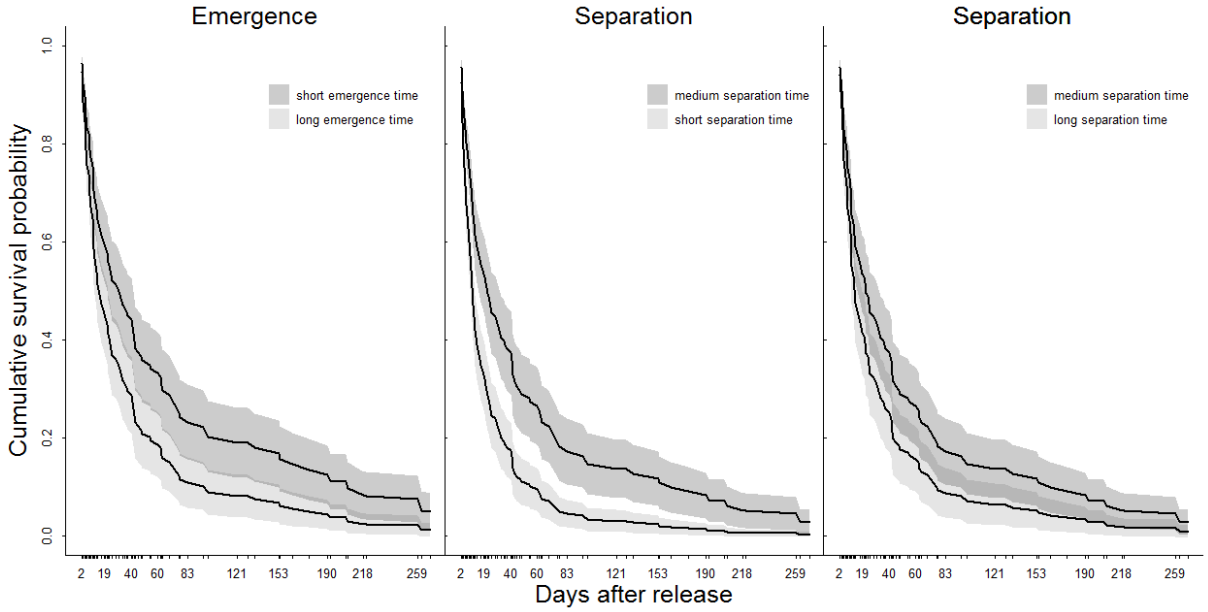

Fig. 5. Estimated cumulative survival and SE over the first 9 months after release as obtained from the final survival model (see last section of results). Individuals showing emergence times of 50 sec (dark shaded curve in left panel) survived longer than birds showing emergence times of 400 sec (light shaded curve in left panel). Birds showing medium separation times of 300 sec (dark shaded curve in middle and right panel) survived longer than birds showing short separation times of 10 sec (light shaded curve in middle panel) or birds that did not return (light shaded curve in panel right).

CHAPTER 4

A lesson for life: food unpredictability in early life increases survival of adult birds after release into the wild

Benjamin Homberger, Lukas Jenni, Jérôme Duplain, Michael Lanz & Michael Schaub

Summary

1. Reintroducing locally extinct species is an important and increasingly used conservation tool. However, many reintroductions fail to establish self-sustaining populations. The quality of the individuals released seems to be a crucial factor affecting reintroduction success, but it is largely unknown which quality traits are important and how they might be improved, especially in captive-reared animals.
2. We developed a pre-release protocol aiming at enhancing post-release survival of captive-bred grey partridges *Perdix perdix*. We experimentally tested the effects of prenatal and postnatal unpredictable food supply on post-release survival of two captive strains (wild and domesticated). 691 full-grown birds representing all eight strain x prenatal x postnatal treatment combinations were released in autumn in 28 social groups (coveys) and followed in the field for six months by radio tracking and visual observations. Data were analysed with multistate capture-recapture models including several random effects.
3. Post-release survival was higher in birds having encountered postnatal unpredictable food supply and decreased drastically with later release dates. Also, coveys strongly affected an individual's survival prospects, while strain, prenatal food supply, sex and year had no substantial effect on survival.
4. *Synthesis and applications*: Post-release survival of captive-bred grey partridges can be increased significantly through simple, inexpensive pre-release measures in captivity, i.e. exposing them to an unpredictable environment. Similar measures might be successful in other species.

Keywords: reintroduction, unpredictable feeding, grey partridge, multistate model, post-release survival, immunity, prepare for release

Introduction

The reintroduction of locally extinct species into their former range has become an important tool in conservation biology (Seddon, Armstrong & Maloney 2007; Seddon, Strauss & Innes 2012). However, re-establishing viable populations of species in the wild is notoriously difficult and the causes of failure often are not fully understood (Kleiman 1989; Wolf et al. 1996; Fischer & Lindenmayer 2000). Given the decline of many species the number of reintroduction attempts will likely increase in the future. Hence, there is an urgent need to better understand the processes that affect reintroduction success (Armstrong & Seddon 2008).

Important factors affecting reintroduction success are the quality of the reintroduction site (i.e. mitigation of the causes of initial decline), the number of individuals released, and the quality of the individuals (Fischer & Lindenmayer 2000). An individual's quality is determined by its origin, i.e. genetic factors, the prenatal and the postnatal environment. Animals originating from wild source populations generally do better after release than animals from captive sources (Fischer & Lindenmayer 2000; Jule, Leaver & Lea 2008). Wild animals appear to have a better physiological setup and a more adequate behavioural arsenal than animals kept in captivity for many generations (McDougall et al. 2006; Frankham 2008; Homberger et al. 2013). However, recruiting animals from wild source populations is often no option since it involves stressful capturing and translocation procedures (Teixeira et al. 2007). Moreover, a sustainable source population from which individuals can be obtained might simply no longer exist. Therefore the release of captive-bred individuals is often the only alternative available. The quality of the individual is further affected prenatally through adaptive parental effects, i.e. parents preparing their offspring for the presumed oncoming environment. For example, birds exposed to stressors (e.g. high predation risk) during ovulation can increase offspring predation evasion ability and flight performance (Coslovsky & Richner 2011). Finally, individual quality is affected by the postnatal conditions (e.g. experiences in captivity, how closely they have been attached to humans) (Mason et al. 2013).

Hence, some studies have sought to optimize rearing methods in order to be able to release individuals of higher quality (Liukkonen-Anttila, Putaala & Hissa 1999). The aim of these efforts is to countervail unwanted effects of captive rearing and breeding and to obtain and release individuals which are able to successfully master all the challenges they

encounter after release. Challenges include the search for suitable structures (e.g. hiding places) and essential resources (e.g. food and water) in the release habitat within a reasonable time span. Further, released animals face predators and parasites to which they are naïve (Sainsbury, Armstrong & Ewen 2012; Ewen et al. 2012) and they have to compete with conspecifics (intraspecific competition) or local niche holders (interspecific competition) (Jule, Leaver & Lea 2008; Moseby et al. 2011). An ideal pre-release preparation would involve procedures that have lasting positive effects on physiology (e.g. physiological stress response, immunity) and behaviour (e.g. anti-predatory behaviour). In addition, pre-release preparations should be inexpensive, easy to conduct and without adverse side-effects.

Studies investigating preparatory measures have often focused on behaviour. Captive training can successfully enhance anti-predatory behaviour in birds (e.g. Gaudioso et al. 2011), fish (e.g. Darwish et al. 2005) and mammals (e.g. Mclean, Lundie-Jenkins & Jarman 1996). Pre-release preparatory measures to counteract negative effects of captivity on physiology and morphology have hardly been tested (Liukkonen-Anttila, Putaala & Hissa 1999), although there are many records showing that captive rearing of wild animals can be accompanied by physiological and morphological alterations which are adverse for survival in the wild (e.g. Putaala & Hissa 1995).

In this study, we developed and tested a pre-release protocol aiming at enhancing the post-release survival of the grey partridge *Perdix perdix* (Linnaeus, 1758) raised in captivity. We investigated whether differences in genetic background, prenatal environment and postnatal rearing conditions affected an individual's ability to survive in the wild after release. For a reintroduction project of this species into Switzerland, we used two captive strains of partridges that varied greatly in the number of generations they have been bred in captivity (2 versus >30; thereafter called wild and domesticated strain). We subjected parental pairs of the two strains and their offspring to periods of unpredictable food supply or *ad libitum* conditions. Unpredictable access to food and water often occurs in the wild and potentially acts as an adaptive cue affecting behaviour and physiology. Finally, when the birds had reached approximately 110 days of age they were released into the wild between September and November in coveys (social groups) that consisted of an equal number of birds from the eight strain x prenatal x postnatal treatment combinations.

We formulated several hypotheses for post-release survival based on the results of a companion study which found that wild strain birds exhibited higher indices of immunity,

oxidative stress resistance, and acute physiological response to stress than domesticated strain birds (Homberger et al. 2013). We expected that these stronger physiological indices would translate into higher post-release survival of wild strain birds. Wild offspring responded to unpredictable feeding of their parents by lowering their acute physiological responses to stress (Homberger et al. 2013). Such parental effects could be adaptive in that they prepare the offspring to better cope with the predicted future environment but they can also result in phenotypic mismatches when prenatal conditions do not reliably predict the postnatal environment (Breuner 2008; Homberger et al. 2013). In either case, we expected that prenatal unpredictable food supply could affect post-release survival positively or negatively. Post natal unpredictable food supply enhanced immune indices irrespective of strain or prenatal conditions (Homberger et al. 2013). We expected that these immune enhancements would increase an individual's ability to cope with pathogenic threats and convey into a higher post-release survival. In addition to the experimental factors we assumed that the sexes could differ in their post-release survival which has been shown repeatedly (Smith & Blumstein 2008). The availability of essential resources and predator pressure changes with time making temporal factors (release date, year) potentially important for survival. Finally, heritable factors (Dingemanse et al. 2004) and the social environment (Watson et al. 2007) could both affect survival, thus we also considered parental pair (family origin) and covey (social group) effects on post-release survival.

Materials and methods

The origin of reintroduced animals

The grey partridge is a ground-dwelling wildfowl species which inhabits arable landscapes in Europa and western Asia (Potts 2012). It is seasonally monogamous and typically raises one clutch per year. After hatching, families form strong bonds which are maintained throughout the breeding season and the subsequent winter and only disintegrate during the consecutive spring (Potts 2012). While historically often present in high densities, many populations in Europe declined strongly in the last decades and in Switzerland the species became virtually extinct at the turn of the millennium (Jenny, Holzgang & Zbinden 2005; Kuijper, Oosterveld & Wymenga 2009). Thus, the Swiss Ornithological Institute started a reintroduction project aiming to re-establish viable populations in Switzerland. The project included measure to improve habitats, and from 2000 onwards grey partridges were released (Buner et al. 2005;

Jenny, Holzgang & Zbinden 2005). In 2009 and 2010 we obtained grey partridge eggs from a UK breeder (Perdix Wildlife Solutions, Warwickshire UK) who held two captive strains. The first strain consisted of male and female birds that were captured from a sustainable wild population on a large game shooting estate in eastern England. Female offspring from these wild pairs were mated with males captured in the wild the consecutive spring. Offspring of this semi-wild strain were subjected to the prenatal treatment (see below) and produced eggs for our study (subsequently called wild strain birds). Birds from the second strain were kept and bred in captivity for over 30 generations and no new wild-caught birds were added (subsequently called domesticated strain). We assumed that the captive population of wild birds genetically closely resembled wild-living partridges, while in the captive strain adaptations to captivity might have occurred (Homberger et al. 2013).

Experimental procedure

In 2009 a total of 22 parental pairs (11 of each strain) and in 2010 50 parental pairs (25 of each strain) were kept in separate outdoor aviaries (3 x 3 x 1.5 m) in the UK (Perdix Wildlife Solutions, Warwickshire UK). Starting one week before oviposition commenced and lasting throughout the laying season (April to June) parental pairs were subjected either to a predictable feeding scheme (subsequently called prenatal predictable food supply) in which they had *ad libitum* access to food (5 pairs per strain in 2009 and 12 pairs per strain in 2010) or access to food was denied during an unpredictable four hour time window between 8 am and 8 pm each day (6 and 13 pairs per strain; subsequently called prenatal unpredictable food supply).

Eggs were collected daily, transported to Switzerland at weekly intervals and artificially incubated. On the hatching day chicks were individually colour-ringed and assigned to indoor aviaries (200 x 80 x 80 cm) with approximately 30 birds per aviary (i.e. 7 or 8 birds per strain x prenatal treatment combination). From age 8 to 43 days (2009) or 8 to 29 days (2010) half of the indoor aviary groups were subjected to a postnatal unpredictable food supply treatment where access to food only (2009) or to food and water (2010) was denied during 3 hours a day at randomly varying time points. The second half of the aviary groups had 24 h *ad libitum* access to food and water. Throughout the first week after hatching and after the postnatal unpredictable feeding regime had ended, all birds had 24 h *ad libitum* access to food (Trutenküken Vormast, Kliba-Nafag, Kaiseraugst, Switzerland) and water. Prenatal and

postnatal food treatments where designed to simulate a temporally unpredictable mild stressor as it frequently occurs in the wild (e.g. bad weather period).

At an age of 29 days birds were relocated into outdoor aviaries (8 x 4 x 2 m) which closely resembled the natural habitat of the species including grassy vegetation, hideaways and sand bathing opportunities. After the postnatal feeding treatment had ended outdoor aviary groups were rearranged into release groups, i.e. release coveys, consisting of approximately four birds per strain x prenatal x postnatal treatment combination. These final coveys were kept together throughout the remaining time in captivity and later released together (see below).

Tagging, release and field observations

A total of 691 individuals in 28 coveys were released between mid-September and mid-November, 2009 (110 individuals) and 2010 (581 individuals). All birds were identifiable by a numbered aluminium ring and three coloured plastic rings. Before release at an age of approximately 110 days all birds were weighted and measured (tarsus length) and 485 birds were equipped with 11 g necklace radio transmitters (<3% of body mass) with mortality switch (RI-2BM; Holohil Systems Ltd., Carp, ON, Canada). Tagging procedures were conducted in accordance with the Guidelines for the Reintroduction of Galliformes for Conservation Purpose (IUCN 2009). The remaining 206 birds were released without radio-tags. Approximately 10 days after tagging birds were transported from the Swiss Ornithological Institute to the release site, the Champagne genevoise, in the south-west of Switzerland (6°04'E, 46°15'N). The release site was the last natural resort of the species in Switzerland, rich in suitable habitats, and was subjected to further habitat improvements in the years preceding the reintroduction project. More than 5% of the 11 km^2 core area consisted of ecological compensation areas of a high quality (Lanz, Michler & Duplain 2012) which are highly important for grey partridges (Buner et al. 2005). We applied a soft-release strategy whereby coveys could acclimatize to the release site for at least 9 hours in release pens on-site where food and water was provided *ad libitum*.

After release the state (live or dead) and location of each radio-tagged individual was recorded at least once per week throughout the observation period using Yagi antennas (Titley Electronics Ltd, Ballina, Australia) and digital receivers (R1000 of Communication specialist Inc, USA and SIKA of Biotrack, UK). Radio-tags were equally distributed among the

eight strain x prenatal x postnatal treatment groups and among coveys. Hence whenever radio-tagged birds were localized in the field we potentially could identify non-radio tagged individuals in this covey by reading their unique colour codes and/or aluminium rings. Thus, all coveys and all covey members, whether radio-tagged or not, received approximately the same localization effort. In addition, large-scale visual censuses were conducted in January following the autumn releases. The whole study area was scoured for grey partridges and colour codes and ring numbers were read whenever possible. In order to maximise the number of radio-tagged birds (which increases detection) we caught and radio-tagged 19 non-tagged individuals between the end of January and the end of March.

Data analysis

Body condition (residual body mass after correction for tarsus length) prior to release was analysed with a general linear mixed model (Bates, Maechler & Bolker 2012) in the statistical software R (R Development Core Team 2012). The main effects of strain, prenatal and postnatal treatments, sex, year and age on body condition were estimated and tested by comparing nested models (including or omitting the focal variable from the model while leaving the rest constant) with likelihood ratio tests (Bolker et al. 2009). Covey and parental pair were added as random effects.

Post-release survival was estimated from visual observations (i.e. colour and aluminium ring identifications), the radio-tag detections and the recovery of dead individuals for a period of six months between mid-September and the end of March, thus the observation time varied depending on release date. The data were summarized in the form of individual capture histories of a length of 14 days. At each encounter one of the following states was assigned to each individual: encountered alive with radio tag, encountered alive without radio tag, captured with radio tag, captured without radio tag, found dead with radio tag, found dead without radio tag, not seen. These capture histories were analysed with a multistate capture-recapture model (Lebreton et al. 2009) that was fitted in a Bayesian framework (Kéry & Schaub 2012). The model provided estimates of biweekly survival probabilities and a number of nuisance parameters such as radio tag loss rate and several detection probabilities. The model is presented in detail in the Supporting Information (Appendix S1). Here we only describe how we modelled survival, since this was our main interest.

Survival was modelled as a linear function of the experimental variables strain, prenatal food treatment and postnatal food treatment. We checked for multiplicative effects of strain and treatment variables by including their interactions (up to the three-way interaction). Moreover, we included effects of time since release and assumed that biweekly survival in the first month after release was different from survival after the first month. Since the experimental treatments may affect survival differently in the two survival periods (first month, later), we considered interactions between the treatments and time since release. We also added release date (Julian date), sex and release year. Finally, we included three random effects: the identity of the release covey (social group), the parental pair (family origin) and the individual. Individual identity was included to account for possible non-independence of individuals and for pseudo-replication (Appendix S1).

We fitted several models to assess how strain, food treatments, their interactions and time since release (first month vs. later) affected biweekly partridge survival. Starting from a model that included all possible effects and interactions, we subsequently fitted models with less parameters. We removed unimportant highest order interactions first and then worked downwards until all unimportant interactions were eliminated. We always maintained the main effects in the model since we considered them biologically important *a priori*. We used effect sizes of interactions to decide whether they were kept or eliminated from the model, i.e. interactions whose 95% credible interval (CRI) included zero were eliminated. We report posterior means and 95% CRI of the parameters of interest. We used vague priors and checked convergence of the Markov chains with the Brooks-Rubin-Gelman statistics. All survival analyses were conducted in WinBUGS (Lunn et al. 2000) run from R via the package R2WinBUGS (Sturtz, Ligges & Gelman 2005). For details on survival modelling showing all fitted models (Appendix T1) and complete WinBUGS code (Appendix S1) refer to the Supporting Information.

Results

Prior to release (at an age of approximately 110 days) body condition (residual body mass) of domesticated birds was higher than that of wild strain birds (estimated mean body condition and CI range for domesticated birds 3.37 g (-6.89 - 13.61), and for wild strain birds -3.77 g (-14.30 - 6.73), LRT = 5.15, d.f. = 1, P = 0.023). Body condition did not differ between birds subjected to prenatal predictable or unpredictable food supply (estimated mean body

condition and CI range for prenatal predictable food supply 3.36 g (-6.88 - 13.61) and prenatal unpredictable food supply= -0.90 g (-11.14 - 9.35), LRT = 1.85, d.f. = 1, P = 0.173). Birds having been subjected to postnatal predictable food supply tended to have a lower body condition than birds encountering postnatal unpredictable food supply (estimated mean body condition and CI range for postnatal predictable food supply group 3.37 g (-6.88 - 13.61) and postnatal unpredictable food supply group= 6.62 g (-3.57 - 16.82), LRT = 3.81, d.f. = 1, P = 0.051). Body condition did not significantly differ between the sexes or between release years (both P-values > 0.4), whereas body condition increased with age (standardized effect size of age ± CI = 2.61 ± 2.40, LRT = 4.53, P = 0.033).

Biweekly post-release survival was not substantially modulated by the interactions of strain, prenatal and postnatal food treatments, thus the final model that we used for inference only contained main effects (Table 1). Mean biweekly radio-tag loss rate was below 1% and the probability to detect a bird was higher if it had been detected at the preceding occasion ('trap happiness'). 'Trap happiness' was evident in birds with and without radio-tags. As expected the detection probability was much higher for radio-tagged birds than for untagged birds. The effect of radio-tags was most evident in the probability to find dead birds. The probability of capture after release was higher for birds without radio tags which demonstrates our effort to catch untagged birds (see Appendix S2 in Supporting Information for the estimates of all nuisance parameters).

Biweekly post-release survival during the first month and later was similar (Table 1) implying consistency in post-release survival throughout the observation period. Survival of wild birds tended to be higher than of domesticated birds but this effect was not substantially different from zero (Fig. 1A, Table 1). There was no substantial effect of prenatal food treatment on post-release survival (Fig. 1B, Table 1) but survival was substantially higher in birds having encountered postnatal unpredictable food supply as compared to birds that were subjected to postnatal predictable food supply (Fig. 1C, Tab. 1). Survival strongly decreased with release date (Fig. 2, Table 1), whereas survival did not differ substantially between the two study years or between males and females (Table 1). The variation in survival was large among coveys as compared to variation among offspring of parental pairs, implying a considerable effect of the social group on individual post-release survival (Fig. 3).

The mean estimated population size and CRI at the end of the six months observation period was 7 (6 – 9) birds of the 110 birds released in 2009 (6.4%) and 44 (37 – 52) birds of the 581 birds released in 2010 (7.6%). The proportion of survivors to the next spring in the eight treatment groups is shown in Fig. 4. Generally, the number of survivors having encountered postnatal unpredictable food supply was more than double that of birds having encountered postnatal predictable food supply. To illustrate the pronounced survival consequences of the combined effects of postnatal treatment and release date and thus to highlight their potential to optimize post-release survival in reintroductions we calculated four marginal cumulative survival curves showing the effects of the postnatal treatments in hypothetical birds released early (mid-September) or late (mid-November) in the release period (Fig. 5). Survival was drastically lower in birds released at the last occasion and the slight differences in biweekly survival between postnatal predictable or unpredictable food supply conveyed into considerable differences in cumulative survival.

Discussion

We investigated whether origin (wild or domesticated strain, parental pair), prenatal and early life experiences (prenatal and postnatal unpredictable food supply) and social context (release covey) affected post-release survival of grey partridges. Contrary to our primary expectations, there were no substantial effects of strain and prenatal food treatment on post-release survival but, as expected, experiencing postnatal unpredictable food supply positively influenced survival after release. Although autumn releases were conducted within a relatively short time frame (mid-September to mid-November), there was a strong negative effect of release date on post-release survival. Finally, whereas prenatal (genetic and common prenatal environment) effects were of minor importance, coveys strongly affected an individuals' survival prospect which highlights the importance of the social group in this species. Our results suggest that captive populations can be prepared to the post-release environment in a simple and low-invasive manner and they emphasize that controllable postnatal factors, namely time point of release and social group structure, can strongly influence survival.

Despite close ancestry to a wild population and physiological advantages presumably beneficial in the wild (Homberger et al. 2013), wild strain grey partridges did not survive substantially longer than birds from the domesticated strain. This may be due to the fact

that adaptations to captivity on multiple levels can occur within very short time frames (e.g. Mason et al. 2013) and could have occurred in our wild strain grey partridges. While such rapid adaptations might help a species to thrive in captivity, they often are detrimental for survival in the wild (Frankham 2008). Keeping and breeding animals in captivity favours traits that accompany the domestication process and typically entails a reduced sensitivity to changes in the environment (Price 1999). In an earlier study, we indeed found evidence for differences in sensitivity to environmental conditions between our grey partridge strains which could have blurred strain differences in post-release survival (Homberger et al. 2013). Wild strain birds exhibited lower physiological responses to acute stress after having encountered prenatal unpredictable food supply as compared to wild strain birds under predictable prenatal feeding conditions. Conversely, the prenatal treatment did not affect the domesticated birds' physiological stress response (Homberger et al. 2013). The high sensitivity of wild (but not of domesticated) strain birds to prenatal conditions suggests that they can adaptively respond to prevailing environmental conditions and thus might have already adjusted to the relatively recent captive environment, e.g. by dampening the physiological stress response (Homberger et al. 2013). However, these recent adaptations could have maladapted the wild strain birds to the demands of a wild environment (Frankham 2008). Body condition of wild strain birds prior to release was lower than in the domesticated strain which could reflect costs associated with these recent adaptation to captivity (Mason et al. 2013). Notably, strain differences in body conditions prior to release did not translate into different post-release survival of wild and domesticated strain birds.

We could not find clear (positive or negative) effects of prenatal unpredictable food supply on post-release survival that would justify or counter its use as a preparatory measure in a reintroduction framework. The prenatal treatment affected the acute physiological stress response of grey partridges (Homberger et al. 2013), but this effect was possibly too weak or not persistent enough to also appear in adult survival after release. While clearly central to physiologically cope with environmental perturbations, the role of the physiological stress response in determining survival is still unclear (Breuner, Patterson & Hahn 2008). A better understanding of the proximal mechanisms underlying such transgenerational effects and how they affect physiological systems could help to more readily predict their fitness-consequences and thus their potential practical value for conservation.

Post-natal unpredictable food supply enhanced post-release survival irrespective of strain and prenatal treatment. Unpredictable food supply and moderate food and caloric restrictions can enhance life span, immunity and supports species-appropriate behaviours (Bloomsmith & Lambeth 1995; Trepanowski et al. 2011). We found that post-natal unpredictable food supply enhanced parts of the innate and the adaptive immune system in grey partridge (Homberger et al. 2013) and tended to increase body condition prior to release. This suggests a direct positive effect of strong immunity on post-release survival which could be associated with high body condition (Bourgeon & Raclot 2006). Pathogens are a major concern within animal reintroductions (Sainsbury, Armstrong & Ewen 2012). Animals to be released have often encountered a limited and irrelevant parasite spectrum in captivity which renders them susceptible to pathogens present on-site (Sainsbury, Armstrong & Ewen 2012). In turn animals weakened by sickness are more prone to predation amplifying pathogen induced mortality (Ewen et al. 2012). The early phases of reintroductions are especially demanding and challenge physiological homeostasis. For example, transportation to the release site is associated with high physiological stress (Teixeira et al. 2007) and high circulating levels of glucocorticoids have well-known suppressive effects on part of the immune system (Sapolsky, Romero & Munck 2000). Dampening the immune system through glucocorticoids makes sense at short term (shifting energy allocation from everyday maintenance to immediate survival). However, if stressors persist for long, for example due to pre-release transportation and handling procedures, glucocorticoids can be chronically increased with detrimental consequences for the animal's immunity and general fitness (Sapolsky, Romero & Munck 2000; Teixeira et al. 2007; Parker et al. 2012).

Beyond the immune enhancing effects, experiencing a phase of unpredictability early after birth may also constitute an enriching environmental stimulus in an otherwise rather monotonous captive environment. Environmental enrichment can increase learning ability and memory function in captive animals (van Praag, Kempermann & Gage 2000) which has stimulated research on animal welfare issues and led to recommendations to enhance the well-being of non-domesticated animals in captivity (e.g. Mason et al. 2007). Importantly, unpredictability is a fundamental characteristic of a new, unknown environment (Parker et al. 2012) and experiencing unpredictability early in life (even in a different context) might essentially prepare the birds to cope with post-release unpredictability. Hence, postnatal

unpredictable food supply could positively stimulate development of a young bird and thus be suitable as pre-release preparatory measure.

We found that survival decreased drastically with release date. This could be due to habitat factors changing with season (e.g. food availability, predation pressure), weather (e.g. decreasing temperature) and higher energetic demands. In our case, day length during the release period decreased by approximately 2 to 4 minutes a day. A bird released in mid-November has therefore around 2 hours less daylight available than a bird released in mid-September and consequently this strictly diurnal species has significantly less time to explore the environment, find adequate habitats and get aware of predators present. A failure to find high quality habitats with low predation risk appears to be a major problem in naïve, reintroduced grey partridges (Rantanen et al. 2010), and our results suggest that maladaptive habitat selection could be even more problematic if released birds have less daytime available to explore the unfamiliar environment.

Our study showed very pronounced differences in survival between release coveys. As such, release coveys might offer manipulative potential to increase post-release survival (Buner et al. 2005; Buner, Browne & Aebischer 2011). Establishing and maintaining an adequate group structure is probably important for many social species and can support Allee effects, i.e positive correlations between population density and individual fitness (Parker et al. 2012). In the grey partridge, group coordination is integral to anti-predatory behaviour or roosting site selection (Tillmann 2009a; Tillmann 2009b). The large variation in post-release survival among coveys could emerge, for example, from differences in personality types of covey members which ultimately affect group stability and flocking behaviour (Webster & Ward 2011). Field records showing that some coveys disintegrate quickly after release or suspicious clustering of death events at the covey level (i.e. after multiple nights without any losses, a predator strikes all of sudden predating several birds at once; JD pers. comm.) could indeed point to inappropriate group coordination impairing adequate anti-predator responses and exposing individuals to high predation risk.

In order to gain control over the post-release environment, a released bird has to collect information on the novel surroundings (e.g. what predators are around, where to find food and shelter). Thereby, social information use is probably an efficient and safe way to gather information given that group decisions are more appropriate than decisions of single birds (Danchin et al. 2004). Experimenting with group compositions, i.e. manipulating group

sizes or proportions of personality types within the group, could provide exciting insights into the inner workings of the social group and reveal ways to enhance reintroduction success (Sih & Watters 2005). We suggest that preparatory measures which support strong group cohesion such as maintaining birth cohorts throughout the time in captivity or establishing family-like bonds by using surrogate parents should ultimately increase reintroduction success (Buner, Browne & Aebischer 2011).

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Table 1. Posterior means and 95% credible intervals (CRIs) of the estimated biweekly post-release survival of grey partridges on the logit scale based on the final model. CRIs of effects that do not include zero indicate variables with substantial effects.

	Post-release survival: final model		
		95% CRI range	
Predictor variables	**Effect sizes**	**lower**	**upper**
Intercept*	0.738	0.199	1.276
Period (after first month)	-0.032	-0.315	0.301
Strain (wild)	0.078	-0.153	0.313
Prenatal treatment (unpredictable)	-0.073	-0.311	0.159
Postnatal treatment (unpredictable)	0.269	0.037	0.505
Sex (male)	0.038	-0.181	0.257
Year (2010)	0.160	-0.394	0.716
Release date	-0.263	-0.473	-0.056

*intercepts shows logit mean survival for level: first month, domesticated strain, prenatal and postnatal predictable food supply, sex female, year 2009 at average standardized values of zero for release date, parental pair and covey.

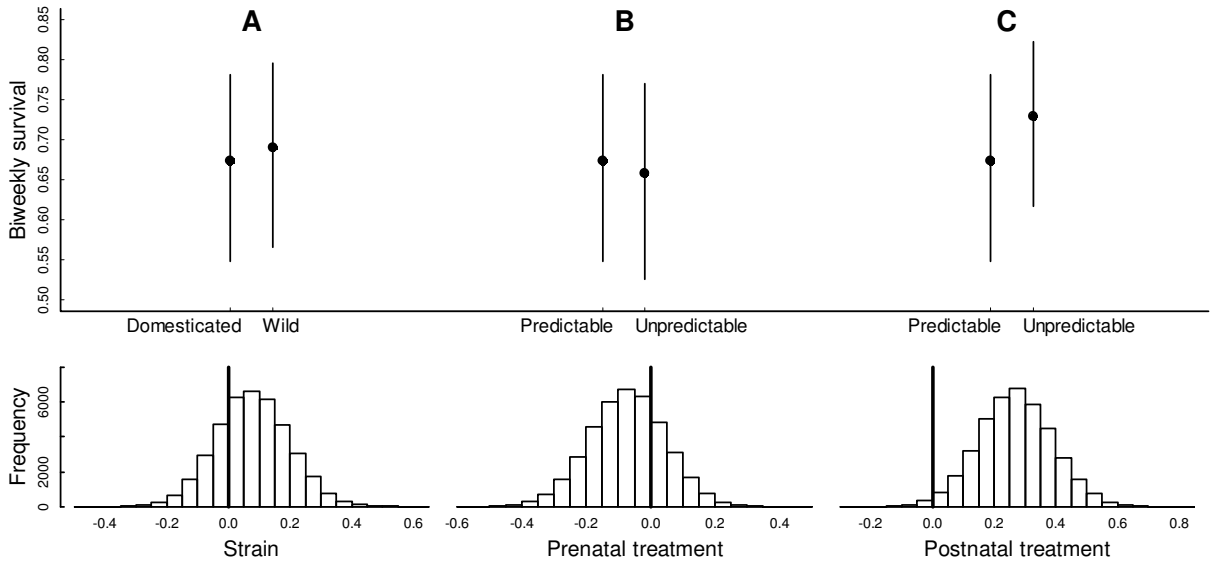

Fig. 1. The upper panels show marginal estimates of biweekly survival in relation to strain (A), prenatal food supply (B) and postnatal food supply (C) and corresponding 95% credible intervals as obtained from the final model. The lower panels show the posterior distributions of the effect sizes of wild strain (left), prenatal unpredictable food supply (middle) and postnatal unpredictable food supply (right) in relation to an effect size of zero (bold lines). The respective non-focal variables are set to first month, domesticated strain, prenatal and postnatal predictable food supply, female, year 2009 and mean release date.

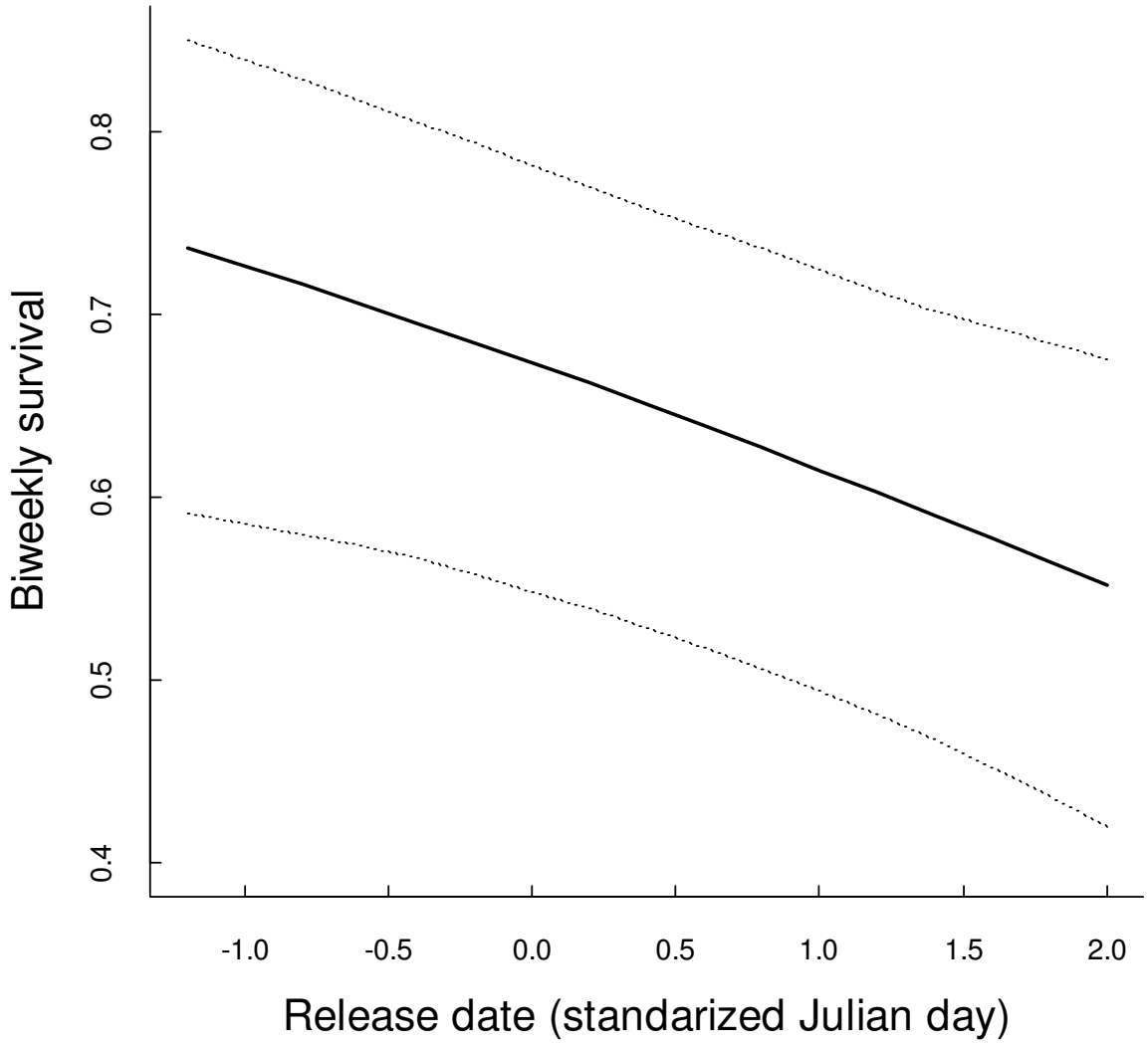

Fig. 2. Estimated effect of release date on marginal post-release survival of grey partridges (straight line) and corresponding 95% credible intervals (dotted lines) obtained from the final model. The x-axis spans the time period during which releases were conducted (mid-September to mid-November). Non-focal variables are set to first month, domesticated strain, prenatal and postnatal predictable food supply, female and year 2009.

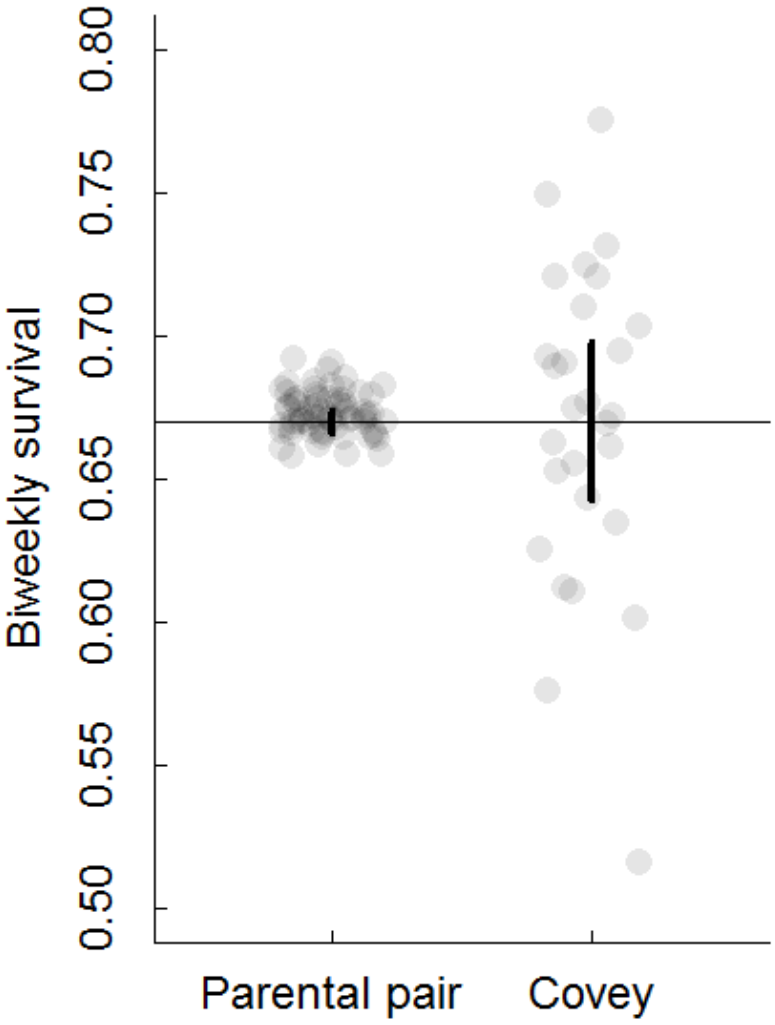

Fig. 3. Variation in mean biweekly post-release survival of grey partridges that originated from different parental pairs and were members in different coveys. The bold vertical lines indicate the standard deviations (i.e. estimated variability between pairs and coveys, respectively). The grey dots are the predicted mean survival probabilities of all the individuals originating from a specific family or covey, thus the number of grey dots is equal to the number of parental pairs and coveys, respectively. The horizontal line indicates overall mean survival. Non-focal variables correspond to the intercept of the final model (Table 1).

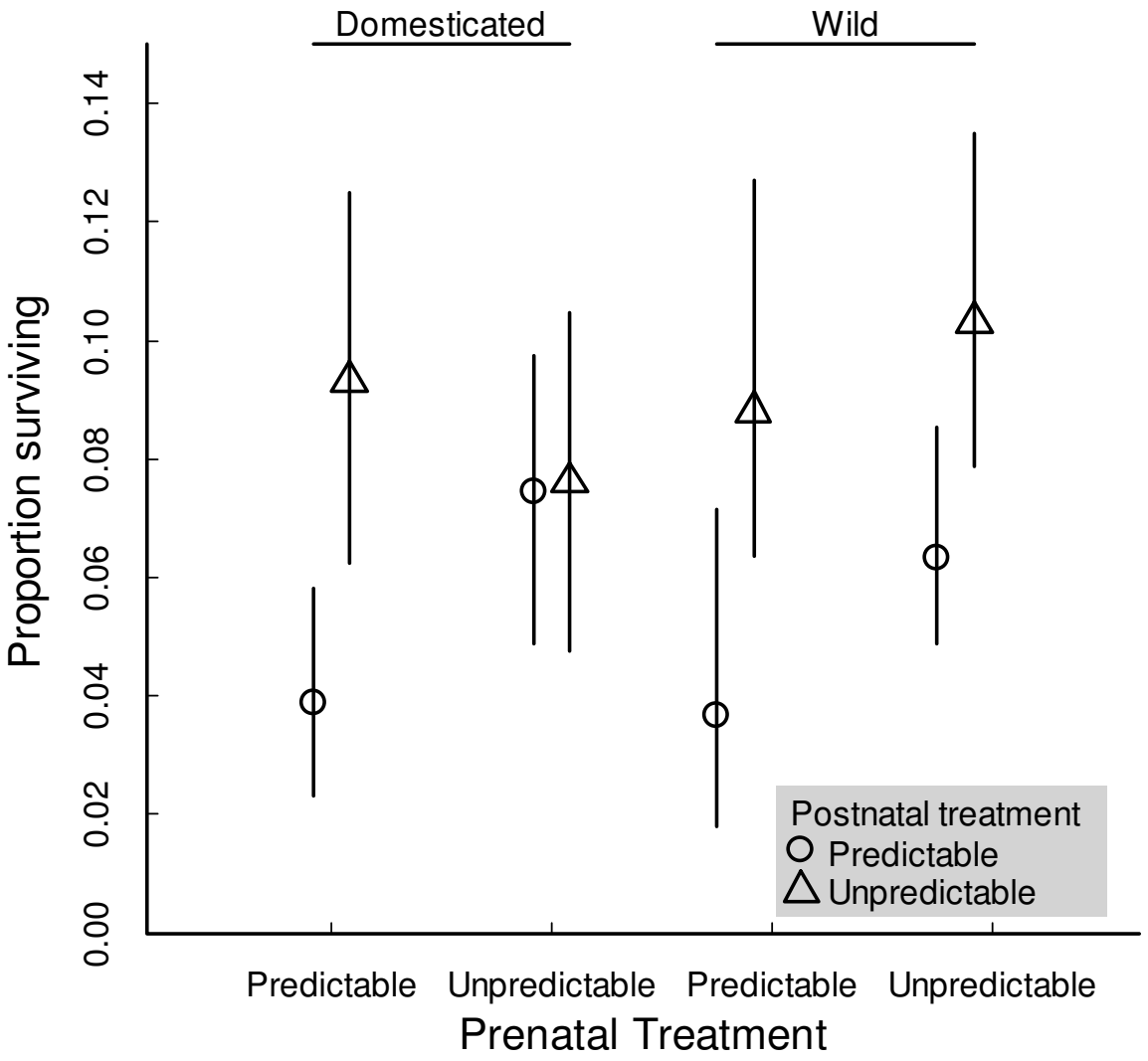

Fig. 4. Estimated proportions and 95% credible intervals of survivors from the eight strain x prenatal x postnatal food supply groups at the end of the six months observation period. The domesticated strain is given on the left side, the wild strain on the right side. Circles indicate postnatal predictable food supply. Triangles indicate postnatal unpredictable food supply. The a-axis indicates the respective prenatal food supply. Estimates are obtained from the final model.

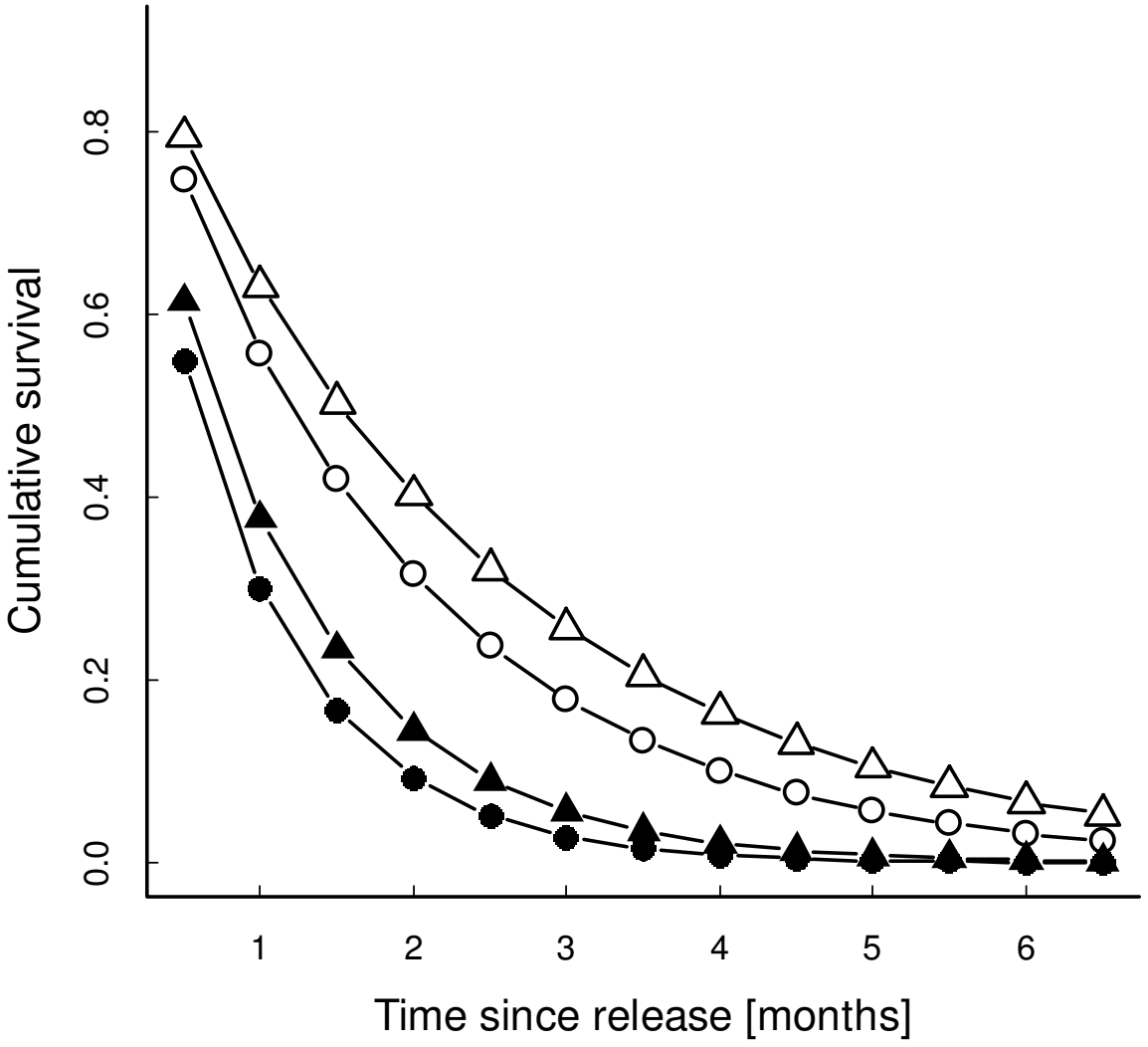

Fig. 5. Cumulative survival throughout time (13 biweekly intervals) for four hypothetical birds having encountered postnatal predictable (circles) or unpredictable (triangles) food supply and released at the earliest (open symbols) or latest (filled symbols) occasion. Covey and pair effects are averaged. Non-focal variables correspond to the intercept of the final model (Table 1).

Supporting Information

Appendix S1. Description of the multistate model.

Table S1. Model selection procedure.

Table S2. Nuisance parameters.

Appendix S1: Description of the multistate model to estimate grey partridge survival

Data preparation

The data are summarized in the form of individual capture histories. At each encounter occasion and for each individual one of seven observational states was assigned. The possible seven states are:

1. seen with a radio tag
2. seen without a radio tag
3. captured with a radio tag
4. captured without a radio tag
5. found dead with a radio tag
6. found dead without radio tag
7. not seen

State 1 (seen with a radio tag) does not necessarily mean that the individual was seen, it was sufficient to get a radio signal indicating that it was alive. The data were originally sampled at a weekly basis, but we analysed them at a biweekly interval to avoid possible boundary estimates of survival. We therefore pooled the observations from two weeks into one occasion. Because it happened that individuals were encountered in two consecutive weeks which we pooled and that their observational states changed over this time, we had to apply priority rules for the pooling. The recovery of a dead individual received priority over all other observations (e.g. when an individual was first seen alive and then recovered dead within the biweekly interval, only the dead recovery was recorded in the capture-history). Finally, capture was given priority over sightings.

We analysed data from a six months period (mid-September to the end of March) from the years 2009/10 and 2010/11. We combined the data sets of the two years vertically and included the release year as a grouping variable. The few individuals released in 2009

which survived until at least September 2010 therefore appear twice in the data set. See in the next section how we adapted the model to avoid pseudo-replication.

Basic structure of the multistate capture-recapture model

The capture histories were analysed with a multistate capture-recapture model (Lebreton et al. 2009). We fitted the model in a Bayesian framework which required its formulation as a state-space model (Kéry & Schaub 2012). Thus, the state transition model describes the development of the true states over time as a first order Markovian process. The observation model links the true states with the observational states. We have already defined the seven observational states above. We consider five true states in the model:

1. alive, with radio tag
2. alive, no radio tag
3. recently dead, with radio tag
4. recently dead, without radio tag
5. dead

The inclusion of three dead states was necessary to model different recovery probabilities for tagged and untagged individuals and to make sure that dead individuals can only be recovered once, right at the occasion after the individual has died. The state transition matrix defines the probabilities that an individual that is in state A at occasion *t* is in state B at occasion *t*+1, and has the following structure:

$$\begin{bmatrix} s_{i,t}(1-\gamma_{i,t}) & s_{i,t}\gamma_{i,t} & (1-s_{i,t})(1-\gamma_{i,t}) & (1-s_{i,t})\gamma_{i,t} & 0 \\ 0 & s_{i,t} & 0 & 1-s_{i,t} & 0 \\ 0 & 0 & 0 & 0 & 1 \\ 0 & 0 & 0 & 0 & 1 \\ 0 & 0 & 0 & 0 & 1 \end{bmatrix}$$

where $s_{i,t}$ is the probability that individual *i* survived from occasion *t* to occasion *t*+1 (survival probability) and $\gamma_{i,t}$ is the probability that individual *i* that had a radio tag at occasion *t* doesn't have his radio tag anymore at occasion *t*+1 (radio tag loss rate). The rows and columns of the state transition matrix refer to the five true states as defined and in the order above.

The observation matrix links the five true states (as defined above) with the seven observation states (as defined above). The true states are in the rows and the observations in the columns of the observation matrix, in the order as defined above:

$$\begin{bmatrix} \left(1-pc_{i,t}^{T}\right)ps_{i,t}^{T} & 0 & pc_{i,t}^{T} & 0 & 0 & 0 & \left(1-pc_{i,t}^{T}\right)\left(1-ps_{i,t}^{T}\right) \\ 0 & \left(1-pc_{i,t}^{U}\right)ps_{i,t}^{U} & 0 & pc_{i,t}^{U} & 0 & 0 & \left(1-pc_{i,t}^{U}\right)\left(1-ps_{i,t}^{U}\right) \\ 0 & 0 & 0 & 0 & r_{i,t}^{T} & 0 & 1-r_{i,t}^{T} \\ 0 & 0 & 0 & 0 & 0 & r_{i,t}^{U} & 1-r_{i,t}^{U} \\ 0 & 0 & 0 & 0 & 0 & 0 & 1 \end{bmatrix}$$

The observation matrix is parameterized with the following parameters:

$pc_{i,t}^{T}$: probability that the radio tagged individual *i* is captured at occasion *t*

$ps_{i,t}^{T}$: probability that the radio tagged individual *i* is resighted at occasion *t*

$pc_{i,t}^{U}$: probability that the untagged individual *i* is captured at occasion *t*

$ps_{i,t}^{U}$: probability that the untagged individual *i* is resighted at occasion *t*

$r_{i,t}^{T}$: probability that the radio tagged individual *i* that died between occasions *t*-1 and *t* is found at occasion *t*

$r_{i,t}^{U}$: probability that the untagged individual *i* that died between occasions *t*-1 and *t* is found at occasion *t*

The model does not account for the possibility that an untagged individual is captured, gets a radio tag and is released as a tagged individual. We haven't found a good way to adapt the model to allow for a deterministic state transition that is conditional on capture. Yet, a simple way to include this possibility without changing the model is to censor the captured individual and to re-release it as a new individual that is now in the tagged state. The inclusion of an individual random effect (see below) accounts for non-independence.

The model specified in the form above assumes that each parameter type could vary by individual and time. Most parameters in this saturated model are not separately estimable and reduced parameter models have to be found. Based on our sampling design, the research questions we are interested in and the biology of grey partridges, we used the following model as our starting model. First, we assumed that most of the nuisance

parameters (radio tag loss rate, recovery probabilities, capture probabilities) were the same for all individuals and that they were constant over time: $\gamma_{i,t} = \overline{\gamma}$, $pc^T_{i,t} = \overline{p}c^T$, $pc^U_{i,t} = \overline{p}c^U$, $r^T_{i,t} = \overline{r}^T$ and $r^U_{i,t} = \overline{r}^U$. Since we captured only between the end of January and March, we fixed the capture probabilities at the other occasions to zero. Secondly, we assumed that the resighting rate (probability to see an individual) could have been subject to immediate trap effects. We specifically supposed the presence of a positive trap response ('trap happiness'), that is, the probability to seen an individual at occasion *t* is larger when it has already been seen at occasion *t*-1 compared to when it was not seen at *t*-1. Such an effect likely depends on the movement of the birds; as long as they stay they are more easily seen. Following Kéry and Schaub (2012) we created the explanatory matrix **K** indicating for each individual whether it has been seen at the occasion *t* ($k_{i,t}$ = 1) or not ($k_{i,t}$ = 2). The resighting probabilities were then $ps^T_{i,t} = \beta^T_{K(i,t-1)}$ and $ps^U_{i,t} = \beta^U_{K(i,t-1)}$. Thirdly, the survival probability was assumed to be a linear function of the release date (x_{date}, Julian date, continuous), time since release (**A**, 2 classes, the first refers to the first month after release, the second to the time afterwards), sex (x_{sex}), year (x_{year}) and the experimental treatment variables strain (x_{str}), pre- (x_{pre}) and postnatal food availability (x_{post}; all of them categorical with two levels). We included interactions between all experimental treatment variables and age, as we wanted to assess whether treatment effects were additive and whether their effect on survival was expressed differentially in the early release phase (first month) as compared to afterwards. Moreover, we added three random effects to estimate the variance components and to account for non-independence. The first one was the release covey (*C*, categorical, 28 levels), we assumed that survival of individuals living together in a covey is not independent. The second one was the parental pair (*P*, categorical, 67 levels). The final one was individual identity (691 levels). This random effect was included to avoid pseudo-replication as some individuals appeared twice in the data set (in year 2009 and 2010) and some were re-released in a different state after physical capture. Thus the linear model for survival is:

$$\begin{aligned}&\text{logit}\left(s_{i,t}\right) = \mu + \varphi_{A(i,t)} + \alpha_1 x_{date(i)} + \alpha_2 x_{str(i)} + \alpha_3 x_{pre(i)} + \alpha_4 x_{post(i)} + \alpha_5 x_{str(i)} A(i,t) + \alpha_6 x_{pre(i)} A(i,t) + \\ &\alpha_7 x_{post(i)} A(i,t) + \alpha_8 x_{str(i)} x_{pre(i)} + \alpha_9 x_{str(i)} x_{post(i)} + \alpha_{10} x_{pre(i)} x_{post(i)} + \alpha_{11} x_{str(i)} x_{pre(i)} x_{post(i)} + \\ &\alpha_{12} x_{str(i)} x_{pre(i)} A(i,t) + \alpha_{13} x_{str(i)} x_{post(i)} A(i,t) + \alpha_{14} x_{pre(i)} x_{post(i)} A(i,t) + \alpha_{15} x_{str(i)} x_{pre(i)} x_{post(i)} A(i,t) + \\ &\alpha_{16} x_{sex(i)} + \alpha_{17} x_{year(i)} + \omega_{C(i)} + \xi_{P(i)} + \varepsilon_i\end{aligned}$$

$$\omega_{C(i)} \sim N\left(0, \sigma^2_\omega\right)$$

$\xi_{P(i)} \sim N\left(0, \sigma_{\xi}^2\right)$

$\varepsilon_i \sim N\left(0, \sigma_{\varepsilon}^2\right)$

where μ is the intercept, φ the main age effect, the $\boldsymbol{\alpha}$ are regression coefficients and σ_{ω}^2, σ_{ξ}^2 and σ_{ε}^2 are the variances measuring the variability between cohort, pairs and individuals, respectively.

Bayesian implementation

The Bayesian implementation of the model requires the specification of prior distributions for all parameters that are estimated. Since we had no a priori knowledge, we opted for vague priors. For μ, φ and the $\boldsymbol{\alpha}$ we used a wide Normal distribution [N(0, 1000)]. We specified uniform priors [U(0,10)] for the standard deviations of the three random effects (Gelman 2006). Finally, for the various nuisance parameters we used uniform priors [U(0,1)].

We fitted the model with software WinBUGS (Lunn et al. 2000) that was executed from R with the R2WinBUGS package (Sturtz, Ligges & Gelman 2005). WinBUGS produces samples from the posterior distributions using Markov Chain Monte Carlo (MCMC) simulations. We generally performed 102'000 iterations, discarded the first 2'000 samples and stored 20'000 samples. The convergence of the Markov chains were evaluated using the R-hat criterion (Brooks & Gelman 1998); it was < 1.05 in all the models.

For a reason unknown to us, WinBUGS could not fit the model that was described above directly, but a reparameterisation of the same model worked (Kéry & Schaub 2012). In this repramaterisation the recoveries of dead individuals were included into the state transition matrix. Thus the state transition matrix was:

$$\begin{bmatrix} s_{i,t}\left(1-\gamma_{i,t}\right) & s_{i,t}\gamma_{i,t} & \left(1-s_{i,t}\right)\left(1-\gamma_{i,t}\right)r_{i,t}^{T} & \left(1-s_{i,t}\right)\gamma_{i,t}r_{i,t}^{U} & \left(1-s_{i,t}\right)\left(\left(1-\gamma_{i,t}\right)\left(1-r_{i,t}^{T}\right)+\gamma_{i,t}\left(1-r_{i,t}^{U}\right)\right) \\ 0 & s_{i,t} & 0 & \left(1-s_{i,t}\right)r_{i,t}^{U} & \left(1-s_{i,t}\right)\left(1-r_{i,t}^{U}\right) \\ 0 & 0 & 0 & 0 & 1 \\ 0 & 0 & 0 & 0 & 1 \\ 0 & 0 & 0 & 0 & 1 \end{bmatrix}$$

and the observation matrix became:

$$\begin{bmatrix} (1-pc_{i,t}^{T})ps_{i,t}^{T} & 0 & pc_{i,t}^{T} & 0 & 0 & 0 & (1-pc_{i,t}^{T})(1-ps_{i,t}^{T}) \\ 0 & (1-pc_{i,t}^{U})ps_{i,t}^{U} & 0 & pc_{i,t}^{U} & 0 & 0 & (1-pc_{i,t}^{U})(1-ps_{i,t}^{U}) \\ 0 & 0 & 0 & 0 & 1 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 1 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 1 \end{bmatrix}.$$

Here is the WinBUGS code for the model:

```
model {

# Linear models for parameters
for (i in 1:nind){
   for (t in f[i]:(n.occasions-1)){
      logit(s[i,t]) <- mu + phi*age[i,t] + alpha[1]*xdate[i] +
alpha[2]*xstr[i] + alpha[3]*xpre[i] + alpha[4]*xpost[i] +
alpha[5]*xstr[i]*age[i,t] + alpha[6]*xpre[i]*age[i,t] +
alpha[7]*xpost[i]*age[i,t] + alpha[8]*xstr[i]* xpre[i] + alpha[9]*xstr[i]*
xpost[i] + alpha[10]*xpre[i]* xpost[i] + alpha[11]*xstr[i]*
xpre[i]*age[i,t] + alpha[12]*xstr[i]* xpost[i]*age[i,t] +
alpha[13]*xpre[i]* xpost[i]*age[i,t] + alpha[14]*xstr[i]*xpre[i]* xpost[i]
+ alpha[15]*xstr[i]*xpre[i]* xpost[i] *age[i,t] + alpha[16]*xsex[i] +
alpha[17]*xyear[i] + omega[cohort[i]] + psi[pair[i]] + eta[ring[i]]

      gamma[i,t] <- mean.gamma
      pcT[i,t] <- mean.pcT[cap[t]]
      pcU[i,t] <- mean.pcU[cap[t]]
      psT[i,t] <- beta1[K[i,t]]
      psU[i,t] <- beta2[K[i,t]]
      rT[i,t] <- mean.rT
      rU[i,t] <- mean.rU
      }
   }

# Priors
mu ~ dnorm(0, 0.001)I(-15,15)
phi ~ dnorm(0, 0.001)I(-15,15)
for (i in 1:17){
   alpha[i] ~ dnorm(0, 0.001)I(-15,15)
   }

for (q in 1:ncohort){
   omega[q] ~ dnorm(0, tau[1])I(-15,15)
  }

for (n in 1:npair){
   psi[n] ~ dnorm(0, tau[2])I(-15,15)
   }

for (r in 1:nring){
   eta[r] ~ dnorm(0, tau[3])I(-15,15)
    }

# SD for random effects
for (i in 1:3){
   sigma[i] ~ dunif(0,10)
   tau[i] <- 1/sigma[i]/sigma[i]
   }

mean.gamma ~ dunif(0,1)
mean.pT[u,1] <- 0
```

```
mean.pT[u,2] ~ dunif(0,1)
mean.pU[u,1] <- 0
mean.pU[u,2] ~ dunif(0,1)
for (u in 1:2){
   beta1[u] ~ dunif(0,1)
   beta2[u] ~ dunif(0,1)
   }
mean.rT ~ dunif(0,1)
mean.rU ~ dunif(0,1)

# Define state-transition and observation matrices
for (i in 1:nind){
# Define probabilities of state S(t+1) given S(t)
   for (t in f[i]:(n.occasions-1)){
      ps[1,i,t,1] <- s[i,t] * (1-gamma[i,t])
      ps[1,i,t,2] <- s[i,t] * gamma[i,t]
      ps[1,i,t,3] <- (1-s[i,t]) * (1-gamma[i,t]) * rT[i,t]
      ps[1,i,t,4] <- (1-s[i,t]) * gamma[i,t] * rU[i,t]
      ps[1,i,t,5] <- (1-s[i,t]) * ((1-gamma[i,t]) * (1-rT[i,t]) +
gamma[i,t] * (1-rU[i,t]))

      ps[2,i,t,1] <- 0
      ps[2,i,t,2] <- s[i,t]
      ps[2,i,t,3] <- 0
      ps[2,i,t,4] <- (1-s[i,t]) * rU[i,t]
      ps[2,i,t,5] <- (1-s[i,t]) * (1-rU[i,t])

      ps[3,i,t,1] <- 0
      ps[3,i,t,2] <- 0
      ps[3,i,t,3] <- 0
      ps[3,i,t,4] <- 0
      ps[3,i,t,5] <- 1

      ps[4,i,t,1] <- 0
      ps[4,i,t,2] <- 0
      ps[4,i,t,3] <- 0
      ps[4,i,t,4] <- 0
      ps[4,i,t,5] <- 1

      ps[5,i,t,1] <- 0
      ps[5,i,t,2] <- 0
      ps[5,i,t,3] <- 0
      ps[5,i,t,4] <- 0
      ps[5,i,t,5] <- 1

# Define probabilities of O(t) given S(t)
      po[1,i,t,1] <- (1-pcT[i,t]) * psT[i,t]
      po[1,i,t,2] <- 0
      po[1,i,t,3] <- pcT[i,t]
      po[1,i,t,4] <- 0
      po[1,i,t,5] <- 0
      po[1,i,t,6] <- 0
      po[1,i,t,7] <- (1-pcT[i,t]) * (1-psT[i,t])

      po[2,i,t,1] <- 0
      po[2,i,t,2] <- (1-pcU[i,t]) * psU[i,t]
      po[2,i,t,3] <- 0
      po[2,i,t,4] <- pcU[i,t]
      po[2,i,t,5] <- 0
      po[2,i,t,6] <- 0
      po[2,i,t,7] <- (1-pcU[i,t]) * (1-psU[i,t])
```

```
      po[3,i,t,1] <- 0
      po[3,i,t,2] <- 0
      po[3,i,t,3] <- 0
      po[3,i,t,4] <- 0
      po[3,i,t,5] <- 1
      po[3,i,t,6] <- 0
      po[3,i,t,7] <- 0

      po[4,i,t,1] <- 0
      po[4,i,t,2] <- 0
      po[4,i,t,3] <- 0
      po[4,i,t,4] <- 0
      po[4,i,t,5] <- 0
      po[4,i,t,6] <- 1
      po[4,i,t,7] <- 0

      po[5,i,t,1] <- 0
      po[5,i,t,2] <- 0
      po[5,i,t,3] <- 0
      po[5,i,t,4] <- 0
      po[5,i,t,5] <- 0
      po[5,i,t,6] <- 0
      po[5,i,t,7] <- 1
      } #t
   } #i

# Likelihood
for (i in 1:nind){
# Define latent state at first capture
   z[i,f[i]] <- y[i,f[i]]
   for (t in (f[i]+1):l[i]){
   # State process: draw S(t) given S(t-1)
      z[i,t] ~ dcat(ps[z[i,t-1],i,t-1,])
      # Observation process: draw O(t) given S(t)
      y[i,t] ~ dcat(po[z[i,t],i,t-1,])
      } #t
   } #i

# Estimate the number of individuals that are alive in the last occasion
for (i in 1:nind){
   t.live[i] <- equals(z[i, l[i]], 1)
   nt.live[i] <- equals(z[i, l[i]], 2)
   }
alive.tagged <- sum(t.live[])
alive.untagged <- sum(nt.live[])
alive.total <- alive.tagged + alive.untagged - 19 # 19 individuals were
censored

} # model
```

The data needed for this model are the matrix with the capture-histories (*y*), all the explanatory variables (***A***, *xstr*, *xpre*, *xpost*, *xsex*, *xyear*), the matrix for the trap-effects modelling (***K***), the sample sizes (*nring*, *npair*, *ncohort*, *nind*, *n.occasions*), a vector indicating in which occasion capture was performed (*cap*), a vector indicating for each individual in which occasion it was marked (*f*) and a vector (*l*) indicating for each individual in which

occasions it was removed (censored) from the sample. Note that most individuals were not censored and thus *l* was the last occasion.

We used the model to estimate the number of individuals that survived until the end of the study. This estimate can be obtained simply by monitoring the latent state variable ***z*** at the last occasion and summing up the state memberships of all individuals.

We were basically interested in the effects of strain, the experimental treatments and the age since release on biweekly survival of the grey partridges. Therefore we looked for a parsimonious structure of the linear model of survival. We performed a backwards modelling whereby parameters whose 95% credible intervals included zero were removed from the model. We started the elimination with the highest order interactions. In total we fitted 4 models, but in the paper we present only the parameter estimates of the simplest one. Yet all fitted models and their parameter estimates are presented in the supplementary Table 1.

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Table S1. Model selection procedure

Model selection procedure showing posterior means and credible intervals of parameters for each step on the logit scale.

	Full model			Reduction step 1			Reduction step 2			Reduction step 3: final model		
		95% CRI range			95% CRI range			95% CRI range			95% CRI range	
Predictor variables	**Effect sizes**	**lower**	**upper**	**Effect sizes**	**lower**	**upper**	**Effect sizes**	**lower**	**upper**	**Effect sizes**	**lower**	**upper**
Intercept[1]	0.726	0.109	1.328	0.732	0.149	1.317	0.671	0.102	1.239	0.738	0.199	1.276
Period (after first month)	-0.110	-0.390	0.633	-0.101	-0.386	0.628	-0.019	-0.277	0.360	0.032	-0.315	0.301
Strain (wild)	0.013	-0.541	0.563	-0.001	-0.468	0.479	0.033	-0.405	0.464	0.078	-0.153	0.313
Prenatal treatment (unpredictable)	-0.012	-0.511	0.472	-0.029	-0.467	0.413	0.111	-0.301	0.518	-0.073	-0.311	0.159
Postnatal treatment (unpredictable)	0.395	-0.080	0.850	0.384	-0.039	0.808	0.293	-0.082	0.669	0.269	0.037	0.505
Sex (male)	0.054	-0.174	0.281	0.053	-0.171	0.279	0.055	-0.176	0.283	0.038	-0.181	0.257
Year (2010)	0.168	-0.417	0.751	0.166	-0.387	0.743	0.181	-0.388	0.760	0.160	-0.394	0.716
Release date	-0.271	-0.489	-0.063	-0.270	-0.489	-0.059	-0.265	-0.488	-0.047	-0.263	-0.473	-0.056
Strain x prenatal treatment	-0.180	-0.872	0.524	0.051	-0.629	0.316	-0.148	-0.639	0.343			
Strain x postnatal treatment	0.198	-0.457	0.870	0.224	-0.224	0.689	0.233	-0.214	0.694			
Prenatal x postnatal treatment	-0.266	-0.834	0.325	-0.249	-0.689	0.195	-0.225	-0.680	0.226			
Strain x period	0.084	-0.369	0.541	0.084	-0.366	0.529						
Prenatal treatment x period	0.307	-0.143	0.757	0.312	-0.129	0.767						
Postnatal treatment x period	-0.168	-0.622	0.283	-0.167	-0.610	0.276						
Strain x prenatal x postnatal treatment	0.041	-0.864	0.939									

[1]intercept is logit mean survival for level: first month, domesticated strain, prenatal and postnatal predictable food supply, female, year 2009 at average standardized values of zero for release date, parental pair, and covey.

Table S2. Nuisance parameters

Posterior means and 95% credible intervals of nuisance parameters obtained from the final model. Given are estimates of biweekly probabilities of losing the tag (tag-loss rate); of the detection probability of radio-tagged bird when they were detected already at the preceding occasion; of the detection probability of radio-tagged bird when they were not detected already at the preceding occasion; of the detection probability of untagged bird when they were detected already at the preceding occasion; of the detection probability of untagged bird when they were not detected already at the preceding occasion; of the probability of capture of radio-tagged or non-radio-tagged birds; and of the probability of recovering a recently dead individual with and without a radio tag.

Nuisance parameters: final model			
		95% CRI range	
Parameters	**Estimates**	**lower**	**upper**
Tag-loss rate	0.009	0.004	0.017
Detection tagged, previously detected	0.841	0.821	0.861
Detection tagged, previously undetected	0.294	0.188	0.414
Detection untagged, previously detected	0.177	0.128	0.235
Detection untagged, previously undetected	0.145	0.104	0.193
Physical capture, tagged	0.079	0.045	0.121
Physical capture, untagged	0.183	0.090	0.313
Tag recovery	0.966	0.937	0.992
Untagged dead bird recovery	0.046	0.021	0.079

CHAPTER 5

Transport and release procedures in reintroduction programs: stress and survival in grey partridges

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Abstract

During translocations stress, as measured by the increase of glucocorticoids, cannot be avoided, but has been suspected to exacerbate the vulnerability to many causes of mortality after release. Therefore, measures to reduce stress have been proposed, such as keeping animals in pens before release (soft-release). In this study, we investigated two open questions in translocations: (a) whether stress caused by the translocation procedure has an effect on survival, and (b) whether soft-release allows recovering from stress induced by capture and transportation. Hand-raised grey partridges showed a moderate adrenocortical response to transportation and kept the capacity to mount a stress response to a new acute stressor, partly by a decrease of corticosteroid-binding globulin capacity. In contrast to studies demonstrating a pervasive effect of capture and transport by virtual elimination of a proper stress response, we demonstrated a robust stress response and a return of baseline levels to pre-transport levels after 33 h of acclimatization. Possibly captive-bred birds may be less sensitive to capture and transportation than wild-caught birds. During the first month after release, birds held 33 h in release pens survived better when their corticosterone levels were lower. However, survival beyond the first month did not differ between birds held 9 h or 33 h in acclimatization pens. Elevated glucocorticoids, as induced by the translocation procedure, likely affect short-term survival after release. We recommend glucocorticoid stress levels be surveyed and minimized during translocations.

Keywords: corticosterone; glucocorticoids; grey partridge; reintroduction; release procedure; stress response; survival; translocation

Introduction

Reintroductions often fail to re-establish a self-sustaining population, which is frequently attributed to the fact that translocated animals survive poorly during the first weeks after release (Wolf *et al.*, 1996; Armstrong & Seddon, 2008; Fischer & Lindenmayer, 2000). Causes for such early failures include predation, starvation, disease and dispersal (Wolf *et al.*, 1996; Letty, Marchandeau & Aubineau, 2007; Dickens, Delehanty & Romero, 2010). Stress induced by the translocation procedure likely exacerbates the vulnerability to many of these direct causes of mortality, e.g. by suppression of the immune system (increased susceptibility to infectious disease), attenuation of the adrenocortical fight-or-flight response (increased susceptibility to predation), increase in energy expenditure and decrease in food intake (increased susceptibility to starvation) (Teixeira *et al.*, 2007; Dickens, Delehanty & Romero, 2009b, 2010). Hence, minimizing stress during translocation could indirectly reduce many direct mortality risks simultaneously.

However, stress during translocation cannot be avoided. All steps of a translocation cause acute stress: capture in the wild or captive environment, handling for inspection and marking, captivity and restraint, transport, acclimatization to the release environment, and release into an unfamiliar environment (reviewed in Teixeira *et al.*, 2007; Dickens *et al.*, 2010; see also Schmidt *et al.*, 2010a,b; Hartup, Olsen & Czekala, 2005). This series of successive acute stress situations often results in a state of chronic stress (Romero, 2004; Dickens *et al.*, 2009b; Dickens *et al.*, 2010). As a consequence the reactivity of the hypothalamo-pituitary-adrenal (HPA) axis may be altered or exhausted, and an adequate adrenocortical stress response to an acute stressor may be dampened or eliminated for at least some time after release (Dickens, Earle & Romero, 2009c). Corticosterone, the main glucocorticoid in birds, circulates either free (probably the biologically active form; Malisch & Breuner, 2010) or bound to corticosteroid-binding globulin (CBG). There is increasing evidence that the environment modulates free corticosterone levels through varying the capacity of CBG (Malisch & Breuner, 2010). Therefore, free corticosterone levels may be elevated through lowering CBG capacity despite a dampened HPA axis. However, to our knowledge CBG capacity and free corticosterone have not yet been measured in any translocation study.

The time until recovery after release from the effects of chronic stress sustained during translocation is virtually unknown (Dickens *et al.*, 2010). Furthermore, there are hardly any

studies investigating whether an altered reactivity of the HPA axis due to stress sustained during translocation has an effect on survival after release and the success of the reintroduction project (Cabezas *et al.*, 2007). Studies that varied the time in captivity or let the animals recover from translocation stress before release and investigated its effect on subsequent survival usually did not measure physiological stress (but see Calvete *et al.*, 2005). Hence, the common assumption that stress induced by translocation actually affects the success of translocations remains largely untested.

Measures proposed to decrease stress during translocation include minimizing the number and time of capture and handling and time of transport, adequate keeping of animals and an acclimatization time before release (Dickens *et al.*, 2010; Letty *et al.*, 2007, Teixeira *et al.*, 2007). Hard-released animals are released immediately after reaching the release site, thereby minimizing time of translocation. Soft-released animals are acclimated at the release site in special enclosures. It is still an open question whether an acclimatization time has positive or negative effects on survival after release. Although some studies have shown a positive effect of soft versus hard release (Bright & Morris, 1994; Carbyn, Armbruster & Mamo, 1994; Letty *et al.*, 2000; Bradley *et al.*, 2005; Tuberville *et al.*, 2005; Devineau *et al.*, 2011), others found no or even a negative effect (Fiechter *et al.*, 1988; Castro *et al.*, 1995; Letty *et al.*, 2000; Thompson *et al.*, 2001; Hardman & Moro, 2006; Siano *et al.*, 2006).

Moreover, it is unclear whether soft-release provides the opportunity to adjust to the new surroundings, whether it provides time to recover from the stress of transportation and handling (Davidson *et al.*, 1997), or whether the transfer to the acclimatization cage elicits another transient stressful situation (Franceschini *et al.*, 2008). There is a need to examine whether soft-release decreases or increases stress and within which time frame, since we know of no study examining stress hormones during hard versus soft release.

In this study, we investigated two open questions in translocations: (a) whether stress caused by the translocation procedure has an effect on the success of translocations and (b) whether soft release allows recovering from stress induced by capture and transportation. We first investigated the adrenocortical response to the different steps of transportation and acclimatization in captive-bred grey partridges (*Perdix perdix*). At each step, we examined whether birds were capable of an adrenocortical response to an acute stressor, and whether they adjusted CBG capacity in order to regulate free corticosterone. We then

examined at the individual level whether corticosterone concentration before release predicted subsequent survival. Since blood-sampling involved additional handling it may have induced additional stress. We thus tested in another experiment with several hundred non-blood-sampled birds whether acclimatization in release pens increased survival after release over a longer time span.

Material and methods

Adrenocortical response to transportation and release and effect on survival

In 2009, eggs of grey partridges were obtained from a breeder in England (D. Butler, Perdix Wildlife Solutions, Warwick, UK) at weekly intervals and incubated in S-chanf, Switzerland. During the first four weeks, chicks were held in indoor pens (10 – 20 individuals on 2 x 0.8 x 0.8 m) and fed a commercial rearing mixture (Trutenküken Vormast, Provimi Kliba AG, Kaiseraugst, Switzerland). At the age of 4 weeks, the chicks were transported to Sempach, Switzerland, assigned to the final release group (covey) together with one adult bird (raised in captivity during the previous year) to reinforce covey cohesion, and housed in groups of 15 - 30 birds in outdoor aviaries of 8 x 4 x 2 m. Aviaries contained short grass, a sandy area for bathing and spruce branches for cover. Water and a commercial food mixture (Parkgeflügel Körner 4225, Meliofeed AG, Herzogenbuchsee, Switzerland) were provided *ad libitum*. During the first 18 weeks, the birds were caught five times in the indoor or outdoor aviaries for examinations (measurements), among them also to take four to five blood samples.

The birds differed in breeding line (second/third generation offspring from wild-caught parents or birds raised since more than 30 generations in captivity). The parents during egg-laying, and the chicks during week 2-4 after hatching, were either held with food and water *ad libitum* or with food unavailable for 4 h at unpredictable times of the day. Breeding line or food conditions of parents or offspring had no effect on baseline or handling-induced corticosterone levels of the chicks in this study and were therefore not included in any analysis.

At the age of 18 weeks, all birds of an aviary (covey) were transported to the Geneva study area (6°04’E, 46°15’N) and released together with the adult bird. Between 29 September and 19 November 2009, seven coveys (207 birds in total, 19 – 38 birds per covey)

were released on five days. A subsample of 76 birds was randomly selected for measuring circulating corticosterone concentration.

Blood samples were collected at four steps of the transportation and release procedure. (a) *Before transportation*: Each covey was caught out of its aviary in the late afternoon and a subsample was blood-sampled. Then, birds were put into transport boxes (60 x 40 x 20 cm) and transported by car to the release site after dusk. After a journey of 3 h they arrived at the release site before midnight. (b) *After transportation*: On arrival, the birds were taken out of the boxes, a subsample was blood-sampled, and all birds of a covey were put in a release-pen (2 x 1 x 1 m). Water and wheat grains were provided in the release-pens but not in the transport boxes. (c) *Before release, 9 h acclimatization*: Four coveys were kept in the release-pens during the rest of the night and a subsample of individuals was blood-sampled between 0800 and 1030 h of the next morning, thus had an acclimatization time of about 9 h in the release pens. (d) *Before release, 33 h acclimatization*: Three coveys were kept in the release pens during the whole next day and following night and a subsample of individuals was blood-sampled between 0800 and 1030 h the next morning, thus had an acclimatization time of about 33 h in the release pens. All birds were released 2–5 h after the last blood-sampling by remotely opening the door of the release pen.

We considered it important to have no effect of the first blood sampling (before transportation) on the second blood sample (after transportation) and to reduce the number of blood samples taken from the same bird. Therefore we used mostly different birds for the first blood sample than for the second, which are both a random sample out of the same pool of birds available. We sampled most individuals both after transportation and before release (44 birds), because we wanted to account for possible between-individual variation, which we thought could be larger as a reaction to transportation than before transportation, and because the birds had to be handled anyhow after transportation and we thought that taking a blood sample would not add much additional stress to this handling. In summary, out of the 76 individuals, 22 birds were blood-sampled only before transportation, 44 birds after transportation and before release, 3 birds only after transportation and 7 birds before and after transportation as well as before release. The number of blood-samples taken on each individual was used as a covariate in the analyses, in order to test if previous blood sampling influenced subsequent corticosterone levels.

At each of the four blood-sampling occasions (a–d), two blood samples were taken. A baseline sample was taken within 3 min after first disturbance (opening the aviary, transportation box or release-pen). We took great care to take the first blood sample within 3 minutes after first disturbance which involved an experienced catching team and several people taking blood samples in the aviaries. This sample thus should be unaffected by catching and handling at this blood-sampling occasion (Müller *et al.*, 2006). The adrenocortical response to an acute stressor (capture and handling of the blood-sampling occasion) was assessed by taking a second blood sample 28 – 40 min after first disturbance. Between the first and second blood sampling, birds were kept in opaque cloth-bags.

Blood samples were taken by puncturing the alar vein and collecting about 40-60 µl blood using heparinized capillary tubes. The blood was centrifuged within 30 min for 5 min and the plasma stored at -20°C. Plasma corticosterone concentration was measured using an enzyme-immunoassay and the affinity and capacity of corticosteroid binding globulin (CBG) was measured with a radioligand-binding assay in the laboratory of the Swiss Ornithological Institute in Sempach (see Appendix S1, Supporting Information).

61 of the 76 individuals were released with a transmitter (RI-2BM Necklace, 11.1 g, with mortality switch, Holohil Systems Ltd., Ontario, Canada) attached to a neck harness (following Buner, Brown & Aebischer, 2011). They were searched in an area of 16 km^2 every third day during the first 4 weeks after release and then once per week. After 218 days all individuals equipped with a transmitter were found dead (57 individuals) or had disappeared (4 individuals). The date of death was taken as the mid-point between the date last recorded alive and the date first recorded dead. In addition, 71 individuals, which belonged to the same 7 coveys, but were not blood-sampled, were also equipped with a transmitter (67 found dead, 4 disappeared). All birds with a transmitter were used to estimate the time after release (in days) when half of the individuals of a covey were dead or had disappeared.

Survival study in 2011-2012

To test whether the time of acclimatization affected subsequent survival without the interference of blood-sampling and radio-tagging, we conducted another experiment. In 2011, eggs were imported from England at weekly intervals, incubated in Sempach, Switzerland, and the chicks raised as in 2009. At the age of 18 weeks, all birds of an aviary were transported to the same study area and released together as a covey including an adult

bird. Between 24 August and 10 November 2011, a total of 33 coveys (653 birds in total) were released on 16 days. These birds were never bled. 16 coveys (314 birds) were released according to the 9 h acclimatization procedure and 17 coveys (339 birds) according to the 33 h acclimatization procedure. The two acclimatization procedures were always alternated to avoid systematic bias with date, release locality or covey size.

All birds were marked with an individual combination of colour rings and none with a radio-transmitter. From December 2011 until February 2012, colour marked birds were searched systematically in the whole area of 10 km^2 where partridges were known to disperse from previous releases of birds with transmitters. Partridge coveys were located acoustically by their calls from 29 fixed points at dusk. Where coveys had been localised, they were searched visually during the next 5 days from a car with binoculars and telescope, in order to identify individuals from their colour rings. Resightings per month were used for the estimation of survival.

Statistical analyses

To assess relationships between different explanatory factors and total corticosterone, free corticosterone and CBG capacity before transportation, after transportation and before release, linear mixed models were used (function lmer; R version 2.10.1, R Development Core Team, 2009). Plasma corticosterone concentration or CBG capacity was the dependent variable. Sex, age, body mass (residuals of the relationship body mass against age), number of blood samples taken before, time after first disturbance (time elapsed between first disturbance and first or second blood sampling, respectively), and release procedure step (a–d) were included in the model as fixed factors, as well as all two-way interactions with release procedure step. Individual, parental pair and covey were included as random factors. Free corticosterone values were increased by 1 and natural logarithm-transformed to obtain normally distributed residuals. Each term of the model was tested by a likelihood ratio test. As none of the two-way interactions were significant all final models contained only main effects. To compare the means between the different release procedure steps (a–d) we used a multilevel modelling approach (Gelman, Hill & Yajima, 2012). Release procedure step was defined as random factor and the shrinked group mean estimates were used to do post-hoc comparisons.

To assess whether corticosterone levels at release were related to survival probability, 6 separate mixed effects Cox proportional hazard models were used (R-package coxme; Therneau 2012). The 4 individuals that disappeared were treated as censored data. The influence of each of the 6 corticosterone measurements (baseline and handling induced total corticosterone, CBG and log-transformed free corticosterone) were analysed in separate models because this enabled us to include the largest possible number of individuals for each analysis and because of collinearity between the 6 variables. Covey was included as a random factor in each model. The significance of the predictors was obtained by z-tests.

For the analysis of the release experiment of colour marked individuals in 2011, we used a Cormack-Jolly-Seber model (Lebreton *et al.*, 1992) to estimate monthly survival and to assess whether survival differed between release procedures (9 h versus 33 h of acclimatization). Preliminary analyses showed that the resighting probabilities in a month were higher for individuals that had already been resighted the month before than for individuals not resighted the previous month (trap-happiness; Pradel, 1993), and that survival in the month immediately after release was lower than in later months. Since individuals within coveys cannot be considered to be independent the release coveys were included as random effects. Because the variability among-coveys might differ between release procedures and between the first month after release and later, we considered four separate random treatment group effects for survival. We also included covey random effects for the resighting probability. However, we assumed that release procedure had no effect on the resighting probability. The estimation of random effects in Cormack-Jolly-Seber models is a challenge within a frequentist framework but is straightforward in the Bayesian framework (Kéry & Schaub, 2012). The Bayesian analysis requires the specification of prior distributions of all parameters and we used flat priors. We used JAGS (Plummer, 2003) to fit the model. We used 3 chains that were run for 30000 iterations each, discharged the first 10000 and kept every 5^{th} sample. Convergence was checked with the R-hat criterion which was < 1.01 for the target parameters.

Results

Baseline total and free corticosterone levels and CBG capacity

Baseline plasma concentration of total and free corticosterone, and baseline CBG capacity were all significantly dependent on release procedure step ($P < 0.001$), but were not significantly affected by time since first disturbance, sex, age, body mass (corrected for age), the number of blood samples taken before, or any two-way interaction with release procedure (Table 1).

Baseline total corticosterone levels after transportation increased significantly over levels before transportation, remained high after 9 h of acclimatization in the release pens and decreased to pre-transportation levels after 33 h of acclimatization (Fig. 1). CBG capacity decreased significantly in response to transportation and during the following 9 h of acclimatization, and approached pre-transportation capacity after 33 h of acclimatization (Fig. 1). Because CBG capacity decreased while total corticosterone increased with transportation, free baseline corticosterone levels were drastically increased after transportation, were still high after 9 h of acclimatization and approached pre-transportation levels after 33 h of acclimatization (Fig. 1).

Handling-induced total and free corticosterone levels and CBG capacity

Handling-induced total corticosterone levels were markedly higher than baseline levels at each of the four release procedure steps, and the same was true for free corticosterone (paired sample t-tests, all *P*-values < 0.001). CBG capacity did not change significantly between baseline and handling (paired sample t-tests, all *P*-values > 0.1).

Handling-induced plasma concentration of total and free corticosterone, and handling-induced CBG capacity were all significantly dependent on release procedure step ($P < 0.02$), but were not significantly affected by time since first disturbance, sex, age, body mass (corrected for age), the number of blood samples taken before, or any two-way interaction with release procedure step (Table 1). Total corticosterone levels attained as a response to handling were significantly lower after transportation than before (Fig. 1). After 9 h of acclimatization handling-induced total corticosterone levels were significantly higher than before and approached pre-transportation levels only after 33 h of acclimatization. CBG capacity decreased significantly in response to transportation and approached pre-

transportation capacity after 9 h and 33 h of acclimatization (Fig. 1). The levels of free corticosterone as a response to handling were similar before and after transportation, rose significantly after 9 h of acclimatization and approached pre-transportation levels after 33 h of acclimatization (Fig. 1).

Survival and corticosterone levels

In the three coveys released after 33 h of acclimatization, survival was related to baseline and to handling-induced corticosterone levels, but not to CBG capacity. Baseline and handling-induced corticosterone concentration were both negatively related to survival (significantly for total corticosterone, almost significantly for free corticosterone; Table 2). Birds with higher total or free baseline or handling-induced corticosterone after 33 h of acclimatization had a lower survival than birds with low corticosterone levels (Fig. 2).

In the four coveys released after 9 h of acclimatization, survival was not significantly related to baseline or handling induced total or free corticosterone concentrations or CBG capacity (Table S1, Supporting Information).

Survival did not differ between the individuals of the 4 coveys released after 9 h acclimatization and of the three coveys released after 33 h (n = 132 birds; hazard ratio = 1.04, LRT = 0.05, df = 1, P = 0.82).

Survival in response to release procedure without prior blood sampling

The estimated monthly survival probabilities of colour marked partridges released in 2011 were lower in the month after release than later, but did not differ significantly between the two release procedures (P = 0.52 and 0.84 for the first and later months, respectively). Survival during the first month was around 0.55 (9 h acclimatization: 0.55 (95% credible interval: 0.31 – 0.80), 33 h acclimatization: 0.54 (0.27 – 0.84)) and around 0.8 after the first month (9 h acclimatization: 0.82 (0.74 – 0.88), 33 h acclimatization: 0.77 (0.69 – 0.85)). Also the variability in survival among coveys was much larger in the first month after release than in later months (9 h acclimatization: variability 40.64 (0.28 – 173.19), 33 h acclimatization: 64.01 (0.87 – 437.80), after first month 9 h acclimatization: 0.20 (0.00 – 1.65), 33 h acclimatization: 0.15 (0.00 – 0.80)).

Discussion

Hand-raised grey partridges showed a pronounced adrenocortical response to handling and a moderate response to transportation. They kept the capacity to mount a stress response to an acute stressor (handling) throughout the whole transportation and acclimatization procedure, partly by a decrease of CBG capacity. Corticosterone levels were near pre-transport levels only after 33 h of acclimatization in release pens. Within the 33 h acclimatization coveys, birds with lower corticosterone levels survived longer after release. However, survival after the first month did not differ between birds held 9 h or 33 h in acclimatization pens.

Adrenocortical response to capture and transport

As consistently found in birds, hand-raised grey partridges elevated their total plasma corticosterone levels about 10-fold (pre-transport) as a response to capture and handling, while CBG capacity decreased only little and plasma free corticosterone level increased dramatically (about 35 fold). Right after transport (when hard-releases would typically be conducted) baseline total corticosterone levels increased only about two-fold over pre-transport baseline levels. This indicates that transportation did not result in high stress-levels, but in moderately increased levels. Elevated total corticosterone after transportation and after the first day of capture was also observed in other birds (Hartup *et al.*, 2005; Dickens *et al.*, 2009c; Nilsson *et al.*, 2008) and mammals (Dickens *et al.*, 2010; Schmidt *et al.*, 2010a,b), but most of these studies suggest that high corticosterone levels were reached, similar to those attained by an acute handling stress. Because CBG capacity decreased as a response to transport, baseline free corticosterone increased about fourfold over pre-transport levels. In the short-term, this may detrimentally affect behaviour and metabolism.

After transportation, partridges were still able to mount a robust, but attenuated adrenocortical response to handling compared to pre-transportation (only a 3-fold increase of total corticosterone). Hence, capture and transportation did not eliminate a proper adrenocortical response to an acute stressor (handling for 30 min), as observed in other studies examining wild birds after transport or taken newly into captivity (Rich & Romero, 2005; Cyr & Romero, 2007; Dickens & Romero, 2009; Dickens *et al.*, 2009c). Interestingly, CBG capacity decreased substantially during transportation and hence baseline free corticosterone was dramatically increased and the level attained as a response to handling

was similar as before transportation. Hence, by decreasing CBG capacity, the response of free corticosterone to handling after transportation was not attenuated, but similar as before transportation.

This supports the hypothesis that corticosterone bound to CBG acts as a reservoir in the plasma which can be mobilised if needed (Malisch & Breuner, 2010). All studies known to us measured total corticosterone as a response to transportation, and thus may have underestimated the increase of the active free corticosterone fraction.

Adrenocortical response and acclimatization time

After 9 h acclimatization, baseline levels of total and free corticosterone and CBG capacity did not differ from post-transport levels. This agrees with other studies which found elevated corticosterone levels after taking birds into captivity for one or several days (Hartup *et al.*, 2005; Dickens *et al.*, 2009c), but horses returned plasma cortisol to pre-transport levels within 2 h after transportation (Schmidt *et al.*, 2010b).

Interestingly, the response of total corticosterone to handling was almost twice as high after 9 h acclimatization and free corticosterone after handling was almost five times higher than before transportation. In contrast to the attenuated or eliminated adrenocortical response to acute handling observed in other studies (Rich & Romero, 2005; Cyr & Romero, 2007; Dickens & Romero, 2009; Dickens *et al.*, 2009c), we found an unprecedented increased adrenocortical response to handling after translocation. There are three possible explanations. First, such a high adrenocortical response to handling may be due to diurnal variation, as blood-samples before and after transportation were taken in the evening and at night, while samples after acclimatization were taken during the morning. We do not think that diurnal variation in corticosterone response explains the high values after 9 h acclimatization, because then we would have expected similar levels after 33 h acclimatization and because other studies found a high stress response at night or at dawn (Breuner, Wingfield & Romero, 1999; Rich & Romero, 2001), while we found the opposite. Second, the very high stress-levels after 9 h acclimatization may be due to the fact that these individuals have all been blood-sampled 9 h before. Rats repeatedly exposed to a certain stressor mount a higher glucocorticoid response to a novel stressor than rats not previously exposed to this stressor, which is termed facilitation (Romero, 2004). Hence transport and the first blood-sampling after transportation may have facilitated a stronger response to the

second handling and blood-sampling event 9 h later. While we cannot exclude this explanation, we think it is less likely because the second blood-sampling is the same stressor as the first (although in different circumstances), and normally similar stressors cause a reduced glucocorticoid response (Romero, 2004). Furthermore, these birds have been blood-sampled already 4-5 times during rearing. Third, the very high adrenocortical response to handling after 9 h acclimatization may have been due to a disrupted negative feedback, i.e. the inability of the HPA axis to reduce corticosterone release sufficiently when plasma levels have reached high levels. This then would indicate a profound, although transient, disturbance of the HPA axis, caused by the series of stressors (transport, handling, putting the birds in the novel environment of the release pen). A disruption of the negative feedback has also been observed in the chukar, although in different contexts (Dickens *et al.*, 2009b,c). An efficient feedback mechanism, however, seems to be vital (Romero, 2004).

After 33 h acclimatization, baseline total and free corticosterone levels were almost back to pre-transport levels and CBG capacity was on its way to regain pre-transport levels. Similarly, the adrenocortical response to handling was about half-way back to pre-transport levels. This indicates that transport and putting birds in release pens were transient stressors and birds were under way to recover by 33 h.

In wild caught chukar, the effects of captivity and returning them back to the capture site or a new site substantially altered stress physiology during at least the first month after release (Dickens *et al.*, 2009b). Baseline levels of total corticosterone, the response to acute handling and the negative feedback response were all lower than immediately after first capture from the wild. It would have been interesting to collect similar data in our captive-bred grey partridges, but recapture after release was unfortunately not possible.

Survival

In birds acclimated for 33 h, when all measures of the HPA axis approached pre-transport levels, we found a negative relationship between total baseline corticosterone or free baseline corticosterone and survival. It is conceivable that high baseline levels are disadvantageous after release, because of the many deleterious effects of corticosterone (e.g. suppressed immunity, elevated metabolism). In the only other study we found about this subject the opposite was found, as baseline plasma and faecal corticosterone metabolite concentrations were positively related to survival in wild-caught rabbits released after 2-4

weeks of captivity (Cabezas *et al.*, 2007). In chukar, recapture rates (possibly representing survival) were similar between birds released immediately at the site of capture, held in captivity and released at the site of capture, and held in captivity and released at a different site (Dickens *et al.*, 2009a), which entailed different degrees of reduced stress response and disturbed negative feedback weeks after release (Dickens *et al.*, 2009b). Possibly, these disparate findings (which remind of the disparate findings regarding the relationship between baseline total corticosterone and survival; Bonier *et al.*, 2009) can be understood if the relationship between baseline glucocorticoids and survival is assumed to be inversely U-shaped (Busch & Hayward, 2009), and the animals of the different studies had baseline corticosterone levels at the increasing or decreasing leg of the curve, respectively.

The high handling response of total or free corticosterone after 33 h acclimatization was apparently not beneficial for survival. We found no other study examining stress-induced corticosterone levels and survival after translocation and there are only a few contradictory studies in wild animals (Breuner, Patterson & Hahn, 2008; MacDougall-Shackleton *et al.*, 2009; Angelier, Holberton & Marra, 2009).

In grey partridges released after 9 h acclimatization, there was no apparent relationship between baseline or handling-induced corticosterone and survival. Whether this is because baseline corticosterone was still elevated in these birds is unclear, as we did not find a difference in survival between 9 h and 33 h acclimatization (but statistical power to detect a difference was low).

Survival in the 2009 experiment was reduced by the transmitters (unpubl. data) and could have been affected by the repeated handling and blood sampling before release. The short survival time (half of the covey members were dead after 3–14 days, depending on covey) precluded us from analysing longer-term effects of the two acclimatization times. Therefore, we tested with new birds in 2011 which were not radio-tagged and not blood-sampled whether survival was affected by acclimatization time. However, this was not the case. There are at least three reasons which may explain why we found no difference in survival between the acclimatization times in 2011. First corticosterone levels may have affected survival only during the first days after release. In the 2009 experiment, 85% of the individuals died within the first month after release and hence a dependence on corticosterone was perhaps more easily detectable; in 2011 55% survived the first month which may have masked short-term effects of corticosterone on survival. Unfortunately, we

could not investigate survival within the first month after release in 2011, because it was impossible to read the colour-marks in the crops before harvest. Second, the coveys differed greatly in survival and this may have masked corticosterone-survival relationships. Third, a 33 h acclimatization time may have made a difference in the 2009 experiment with the added stress of blood-sampling and transmitter, but not in 2011 when a longer acclimatization time would possibly have been more appropriate.

Conclusions

Efforts to reintroduce grey partridges and other game birds are plentiful. Breeding, rearing and releasing methods have been evaluated in detail (e.g. Buner *et al.*, 2011; Buner & Schaub, 2008). However, the effects of stress during translocation have only been investigated in one study which showed that the HPA axis is altered even weeks after release (Dickens *et al.*, 2009b).

From our study it appears that hard-release or a short acclimatization time (i.e. 9 h) would entail moderately elevated levels of circulating corticosterone and possibly a disturbed negative feedback. In contrast to studies demonstrating a pervasive effect of capture and transport by virtual elimination of a proper stress response, we found a robust stress response and a return of baseline levels to pre-transport levels after 33 h of acclimatization. Possibly captive bred birds are less sensitive to translocation compared to the wild-caught birds used in the published studies (Rich & Romero, 2005; Cyr & Romero, 2007; Dickens & Romero, 2009; Dickens *et al.*, 2009c; see also Adams *et al.*, 2005). In captive-bred whooping cranes (*Grus americana*), corticosterone also returned to normal levels within one week after transportation to another site (Hartup *et al.*, 2005).

We found evidence that elevated baseline levels, as they are unavoidably induced by translocation, reduce subsequent short-term survival. Therefore, we recommend glucocorticoid levels be surveyed and stress minimized during translocations. From the disparate findings in the literature, it appears that measures to minimize stress have to be evaluated for each species until more generalized patterns can be discerned. Therefore, in all studies which monitor the fate of released animals individually, it would be very valuable to measure glucocorticoids before release.

An acclimatization time of 33 h was most likely too short to regain a proper HPA-axis functionality. Further studies should investigate whether a longer acclimatization period

would take handling-induced corticosterone levels back to pre-transport levels and result in higher survival. However, a longer acclimatization time in the release pens increases other risks such as injury, diseases and the attraction of predators (Buner & Aebischer, 2008; own experience). Our study also showed that putting the birds in release pens is perceived as a transient stressor and needs an additional acclimatization time. Therefore, we recommend evaluating carefully whether soft-release is indeed beneficial and what acclimatization time is needed to balance the benefits and disadvantages of soft-release.

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Table 1. Dependence of baseline and handling-induced total corticosterone, CBG capacity and free corticosterone levels (ln-transformed+1) on the explanatory variables time after first disturbance, sex, age at day of transportation, body mass (corrected for age), number of blood samples taken before, and release procedure step as fixed effects. Individual, release covey and parental pair were included as random intercepts in a linear mixed model. For each of the three separate linear mixed models, the likelihood ratio test (LRT), degrees of freedom (df) and p-values are given for all fixed effects. Significant terms are highlighted in bold. All interactions with release procedure step were not significant and removed from the model.

	Total corticosterone (ng/ml)			**CBG capacity (nM)**			**Ln (free corticosterone+1) (ng/ml)**		
Explanatory variable	LRT	df	p	LRT	df	p	LRT	df	p
Baseline									
Time after first disturbance	2.74	1	0.1	0.05	1	0.82	0.89	1	0.35
Sex	0.1	1	0.75	0.3	1	0.13	0	1	1
Age	0.55	1	0.46	0.31	1	0.58	0	1	1
Body mass	0.5	1	0.48	1.47	1	0.23	0.01	1	0.91
Nb. blood samples taken before	1.26	1	0.26	0.21	1	0.27	0.2	1	0.65
Release procedure step	38.45	3	**< 0.001**	54.69	3	**< 0.001**	37.61	3	**< 0.001**
Handling-induced									
Time after first disturbance	0	1	1	0	1	1	0	1	1
Sex	0.29	1	0.59	2.24	1	0.13	1.07	1	0.3
Age	0	1	1	0	1	1	0.17	1	0.68
Body mass	0.12	1	0.73	0.69	1	0.41	0.5	1	0.48
Nb. blood samples taken before	0.42	1	0.52	2.4	1	0.12	3.53	1	0.06
Release procedure step	23.73	3	**< 0.001**	9.64	3	**0.02**	25.65	3	**< 0.001**

Table 2. Relationship between survival time (in d) as the dependent variable, covey as random factor, and baseline or handling-induced total corticosterone, CBG capacity or free corticosterone (ln-transformed) as explanatory variables analysed in six separate mixed effects Cox regressions for grey partridges acclimated for 33 h. Each row indicates one model. Columns 2, 3, and 4 give the exponential of the coefficient (hazard ratio) for the corticosterone variable and their test statistics, and the last column gives the between-covey variance. Sample size was 21 birds, except for baseline total corticosterone (n = 20) and baseline CBG and free corticosterone (n = 19).

	Corticosterone or CBG			**Covey**
	Exp(coefficient)	Z	p	Variance
Baseline				
Model with total corticosterone	1.28	2.68	0.007	0.0005
Model with CBG capacity	1.01	1.44	0.15	0.0001
Model with free corticosterone	60781.6	1.7	0.089	6.634
Handling-induced				
Model with total corticosterone	1.04	1.96	0.051	0.0002
Model with CBG capacity	0.99	-1.41	0.16	0.0001
Model with free corticosterone	2.43	1.82	0.069	0.4039

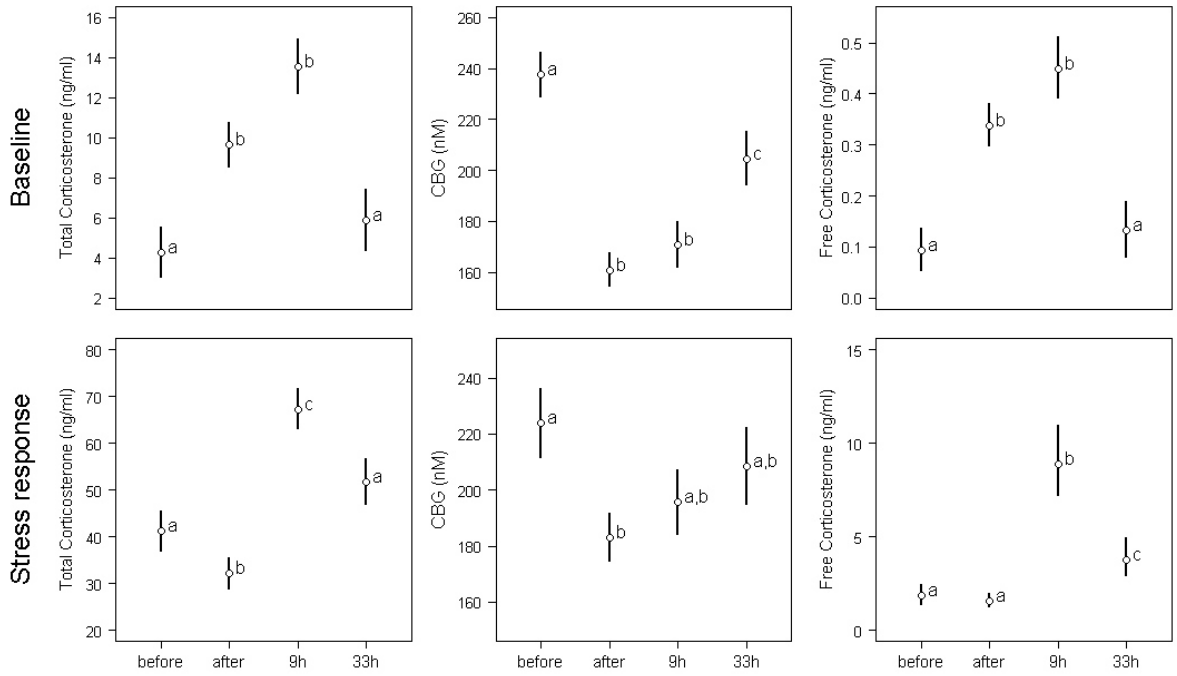

Fig. 1. Plasma concentration of total and free corticosterone and CBG capacity for the four release steps: before transport, after transport, after 9 h acclimatization and after 33 h acclimatization. Baseline values are given in the upper row, handling-induced stress responses are given in the lower row. Mean values (± SE) predicted by the models given in the Electronic Appendix 2 are indicated. Predicted means are significantly different from each other ($p < 0.05$) when they do not share the same letters (see Statistical analyses).

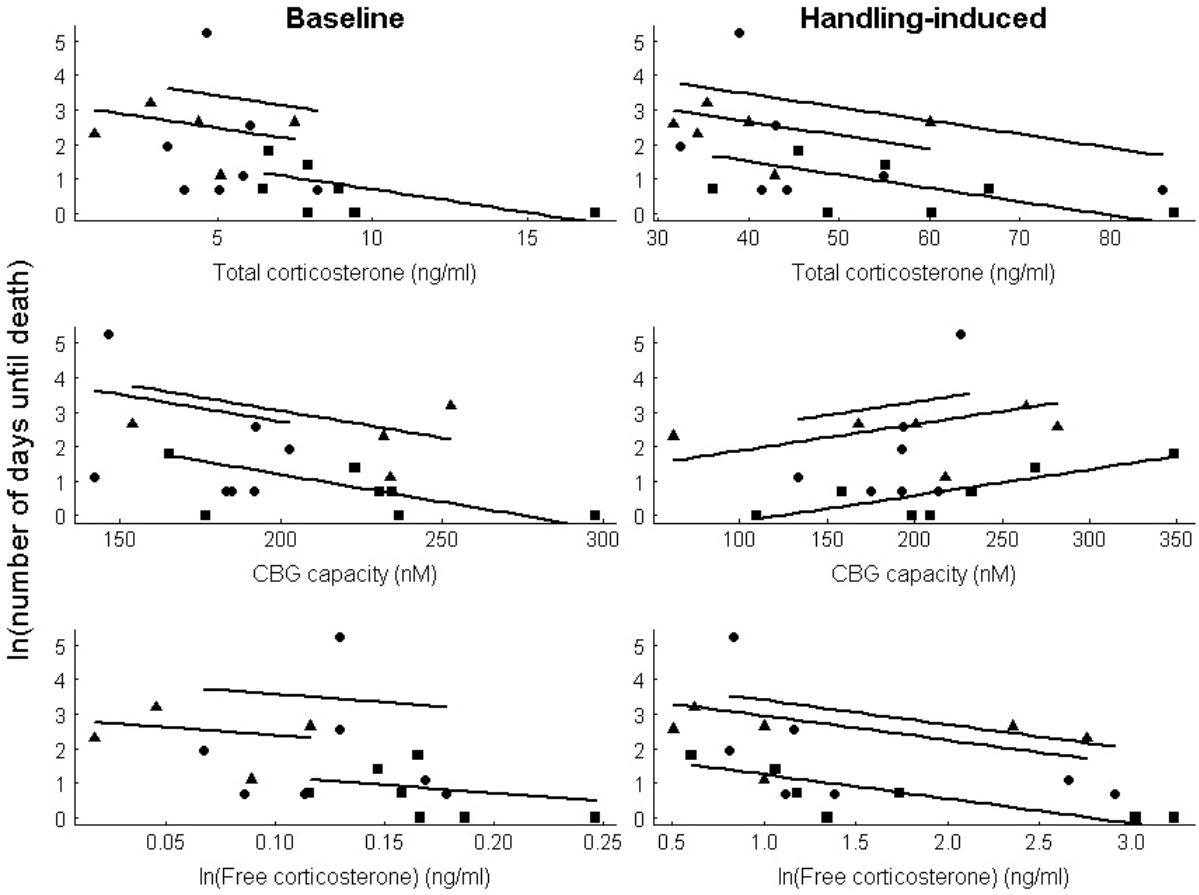

Fig. 2. Number of days until death (in d, ln-transformed) in relation to total corticosterone, CBG capacity and free corticosterone at baseline (left column) and after handling stress (right column). Symbols represent the three coveys held during 33 h in the release pen which took 3 d (dots), 4 d (triangles) and 14 d (squares) until half of the covey members with transmitters were dead or had disappeared. Lines indicate the expected logarithm of survival time per covey obtained from a model assuming constant survival probability over time (exponential distribution of survival time).

Supporting information

Appendix S1. Determination of total corticosterone, corticosteroid binding globulin and free corticosterone.

Table S1. Relationship between survival and glucocorticoids in the four coveys of grey partridges released after 9 h of acclimatization.

Appendix S1: Determination of total corticosterone, corticosteroid binding globulin and free corticosterone

Total corticosterone assay

Plasma corticosterone concentration was measured using an enzyme-immunoassay (EIA; Munro and Stabenfeldt, 1984; Munro and Lasley, 1988) in the laboratory of the Swiss Ornithological Institute in Sempach. Corticosterone in 5 µl plasma and 195 µl water (H_2O_{bidest}) was extracted with 4 ml dichlormethane, re-dissolved in phosphate buffer and measured in triplicates in the enzyme-immunoassay. The dilution of the corticosterone antibody (Chemicon; cross reactivity: 11-dehydrocorticosterone 0.35 % , progesterone 0.004 %, 18-OH-DOC 0.01 %, cortisol 0.12 %, 18-OH-B 0.02 % and aldosterone 0.06 %) was 1:8'000. HRP (horseradish peroxidase, 1:400'000) linked to corticosterone served as enzyme label and 2,2'Azino-*bis*(3-ethylbenzo-thiazoline-6-sulfonicacid)diammonium salt (ABTS) as substrate. The concentration of corticosterone in plasma samples was calculated by using a standard curve run in duplicates on each plate. Plasma pools from chicken with two different corticosterone concentrations were included as internal controls on each plate. Altogether 18 plates were run on 5 different days. The detection limit of the assay was 1 ng/ml. If the concentration was below detection threshold, the value of the lowest detectable concentration (1 ng ml^{-1}) was assigned (5 samples). Intra-assay variation ranged from 5.04 % to 11.27 %, inter-assay variation from 5.72 % to 12.12 % depending on the internal controls.

Corticosteroid binding globulin and free corticosterone

The affinity and capacity of corticosteroid binding globulin (CBG) was measured with a radiogland-binding assay with tritiated corticosterone following Breuner and Hahn (2003).

Briefly, 5 µl plasma was incubated with dextran-coated charcoal solution (0.1% dextran, 1% Norit A charcoal in 50 mM Tris) 20 min at room temperature to strip endogenous steroids. Plasma dilution was optimized for grey partridges yielding a solution of 1: 900 and an incubation period of 2 h. Except during the stripping process, plasma and all assay buffers were maintained at 4°C. All samples were run in triplicates. Total binding was determined using 50 µl buffer (50 mM Tris), 50 µl ^{3H}corticosterone (20 nM ^{3H}cort) and 50 µl stripped plasma. Non-specific binding was determined using 50 µl unlabelled corticosterone (1 µM cort) instead of buffer. Glass fibre filters were soaked in 25 mM Tris with 0.3% polyethyleneimine for 1 h before vacuum filtration (Brandel Harvester). Filters were rapidly rinsed with three rinses of 3 ml ice-cold 25 mM Tris. Following filtration radioactivity bound to filters was measured by standard liquid scintillation spectroscopy (scintillation cocktail Ultima Gold^{TM}. LLT, Perkin Elmer). The equilibrium binding parameters for the specific binding of ^{3H}cort were determined through equilibrium saturation binding assay of pooled grey partridge plasma and ^{3H}cortconcentration between 0.25 and 12.0 nM. Affinity estimates (dissociation constant K_d) of corticosterone for CBG in grey partridge were 1.45 nM and the maximal binding sites (B_{max}) 189.7 nM (see Fig. A1). Individual hormone binding capacity was estimated using point sample analysis, that is, measuring CBG capacity using one concentration of ^{3H}cort. Percentage CBG bound in the assay was estimated using the following formula: % bound = [^{3H}cort] / ([^{3H}cort] + K_d). For analysis all point samples were corrected to 100%. A plasma standard was included in all CBG assays which yielded intra-assay coefficient of variation of 16.85% and inter-assay coefficient of variation of 5.84 %.

Free corticosterone levels were estimated from total corticosterone concentrations and CBG binding parameters by use of the equation of Barsano and Baumann (1989):

$$H_{free} = 0.5 \times \left[H_{total} - B_{max} - \frac{1}{K_a} \pm \sqrt{\left(B_{max} - H_{total} + \frac{1}{K_a} \right)^2 - 4 \times \left(\frac{H_{total}}{K_a} \right)} \right]$$

H_{free} is free hormone, H_{total} is total hormone, B_{max} is total binding capacity of CBG, and K_a = 1/dissociation constant (K_d) (all values in nM).

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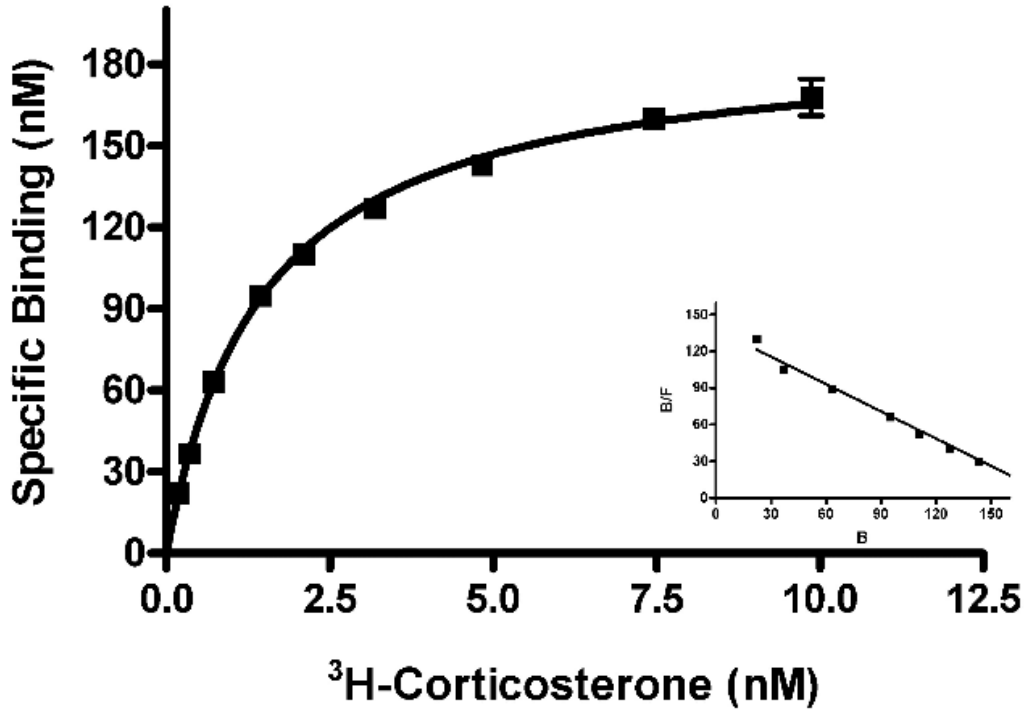

Fig. A1. Equilibrium saturation binding curve demonstrating specific binding of ^{3}H-corticosterone to grey partridge plasma as a function of increasing concentrations of radiolabeled corticosterone. Points represent means ± SE. The inlay is the Scatchard-Rosenthal replot of the data, with B = bound- and F = free- ^{3}H corticosterone fraction.

Table S1. Relationship between survival time (in days) as the dependent variable, covey as random factor, and baseline or handling-induced total corticosterone, CBG capacity or free corticosterone (ln-transformed) as explanatory variables analysed in six separate mixed effects Cox regressions for grey partridges acclimated for 9 h. Each row indicates one model. Columns 2, 3, and 4 give the exponential of the coefficient (hazard ratio) for the corticosterone variable and their test statistics, and the last column gives the between-covey variance. Sample size was 23 birds for baseline total corticosterone, 22 birds for baseline CBG capacity and baseline free corticosterone, 25 birds for handling-induced total corticosterone and 24 birds for handling-induced CBG capacity and handling-induced free corticosterone.

	Corticosterone or CBG			**Covey**
	Exp(coefficient)	Z	p	Variance
Baseline				
Model with total corticosterone	0.99	-0.12	0.9	2×10^{-6}
Model with CBG capacity	0.98	-0.98	0.33	1×10^{-8}
Model with free corticosterone	1.73	0.44	0.66	2.5×10^{-3}
Handling-induced				
Model with total corticosterone	0.99	-0.54	0.59	2×10^{-7}
Model with CBG capacity	1	0.16	0.88	5×10^{-9}
Model with free corticosterone	0.91	-0.38	0.71	8×10^{-5}

SYNTHESIS

This thesis tackles two main research goals which are interesting from a purely scientific perspective and which could also have implications for animal reintroduction projects. First, we aimed to elucidate how phenotypic expression of two strains of grey partridges is shaped by two prenatal and postnatal contexts, i.e. periods of prenatal and postnatal unpredictable food supply (Chapters one, two and three). Second, we explored the survival differences both between the two strains and in regard to the perinatal feeding schemes and behaviour (Chapters three and four). In the additional chapter five we investigated how pre-release translocation procedures affected stress hormone levels and whether acclimatisation at the release site has positive effects on the stress state and on post-release survival. Throughout the study we also considered other potentially important factors such as reversible phenotypic flexibility within individuals or the resemblance of phenotypic traits and survival among full siblings, i.e. offspring of the same parents, and similarities between covey members, i.e. members of the same social group.

I draw two main conclusions from this thesis. First, grey partridges exhibited substantial phenotypic variability which was founded not only on their genetic heritage but also on the ability to incorporate and translate the prenatal and postnatal environmental conditions into phenotypic variability. Indeed, the steroid hormone corticosterone appears to be a key factor at the interface of the internal physiological milieu and the external environmental conditions. Second, it was possible to modulate the captive rearing conditions through a simple measure in such a way that post-release survival of grey partridges was enhanced. If similar measures prove successful in other species, they could ultimately aid animal reintroductions and conservation.

Below, I focus on several aspects of the thesis which I found especially interesting. First, there were indeed considerable strain differences in important physiological systems which, however, did not translate into post-release survival differences. Compared to the domesticated strain, wild strain grey partridges showed stronger immune indices, higher oxidative stress resistance and a more pronounced short-term (up to 250 seconds) and medium-term (around 30 min) corticosterone excretion in response to an acute stressor. These results imply that wild strain grey partridges might be physiologically better adapted to cope with wild environments as compared to domesticated birds, but this was not evident at least when considering survival as the measure of fitness.

Apparently, animals adapted to captive environments do not fit the post-release environment. Hence, there exists a mismatch between phenotypic attributes adaptive in the current captive vs. the future wild environment (Frankham 2008; Mason *et al.* 2013). Selection in captivity is biased toward 'captivity phenotypes' (Swaisgood 2010). Our wild strain grey partridges, which were comparatively recently adapted to a wild environment, might still be in the middle of a process of adapting to their more recent captive conditions. Suddenly, however, these wild strain birds once again encounter a substantially different post-release environment. Keeping this adaptation history in mind, I find it more understandable that they cannot readily translate physiological advantages into higher post-release survival.

This leads me to a second interesting aspect of the thesis; the diverging effects of the prenatal vs. the postnatal unpredictable food supply or more figuratively, the cautious prenatal expectations vs. the hard postnatal reality. There was some evidence for adaptive physiological adjustments induced by prenatal unpredictable food supply which were most evident in the corticosterone response to a stressor. These physiological adaptations were differently affected by strain and probably also differed between the two study years. Therefore, prenatal unpredictable food supply triggered subtle mechanisms to fine-tune physiology according to the prevailing conditions but there are likely to be more important but unmeasured factors constituting the internal milieu and determining homeostatic regulations. Consequently, from an applied perspective the inconsistent prenatal effects are not reliable and are thus impractical as tools to (advantageously) modify phenotypes for release. On the other hand, postnatal unpredictable food supply did enhance immune indices in both strains, irrespective of prenatal conditions, and it also entailed a higher post-release survival. The survival benefits could be related to the immune 'reinforcements', but also the experience of non-predictable conditions in early life could ultimately have prepared the birds for the completely new and unpredictable post-release environment. Hence, the effects of postnatal treatment were more predictable and recognisable in post-release survival, which suggests their practical use for conservation and also highlights the great importance of the postnatal environment in adapting the phenotype. Post-release survival rates (and thus the absolute number of survivors) were low as is to be expected in a reintroduction context (Buner, Browne & Aebischer 2011). Likewise the positive effect of the postnatal treatment on survival could appear marginal. As yet, considering the similarly low

juvenile survival rates of many wild living bird species including the precocial grey partridge with its large clutch size (Potts & Aebischer 1995; Panek 2006) it is actually a more than doubling of the survival rate, which is quite remarkable (Chapter 4).

Changes of the (prenatal) embryonic milieu can quickly become detrimental to the developing embryo (Sapolsky, Romero & Munck 2000). Thus, the role of maternal effects could be one of careful physiological fine-tuning within the narrow boundaries of physiological homeostasis. In contrast, substantial adaptations of the postnatal phenotype are vital, potentially less risky to normal development and common in many species. For example, a reduction of the physiological response to stress induced by prenatal unpredictable food supply could well support physiological health and longevity in a captive setting. However, for survival in a reintroduction context it is presumably more important to behave appropriately, e.g. synchronize individual behaviour to the social group even if this incurs social stress. Hence, in a severe post-release context appropriate behaviour is presumably much more important than appropriate physiological fine-tuning. This notion is supported by strong postnatal effects on survival. For example, survival strongly decreased with later release dates, which implies that the post-release environment underwent profound changes during the short time span when releases were carried out. Only individuals which can quickly adjust their physiology and behaviour might be able cope with these rapid environmental changes (Piersma & Drent 2003).

The strong covey effects revealed in this thesis are a final remarkable aspect of the postnatal (social) environment. Covey effects were evident from a basic physiological stress level up to individual behaviour, and even post-release survival differed substantially between the coveys. The fitness benefits of a gregarious life, which are well known in general, must be very important in the grey partridge, vitally linking an individual's life expectancy to the welfare of its social group. On the other hand, the fact that phenotypic traits are more similar within than among coveys (within-covey variation is lower than among-covey variation) even implies that selection and evolution could occur at the level of the covey (Wilson & Wilson 2007). Having repeatedly observed grey partridges in the field I have no doubt that group life is of outstanding biological importance in this typical prey species. The way individuals move, feed and flush as a whole appears dizzying to the spectator but is indeed well coordinated and the perils for the single individual deviating from this covey unity become obvious. Understanding the factors that underlie this covey

unity and actively promoting them in a captive setting could ultimately enhance the success of many reintroduced social species.

In summary, the chapters of this thesis cover a large range of topics from individual physiological and behavioural phenotypic expression to post-release fitness. The chance to work in an applied reintroduction framework allowed the implementation of a thorough experimental design and the formulation of research questions relevant for pure and applied science alike. The use of state-of-the-art statistical techniques unlocked the full potential inherent in the data and enabled the adequate testing of a set of a priori hypotheses. Indeed, more questions ensue, for example, how does corticosterone directly affect fitness and what is its role as a mediator of parental effects. These questions clearly merit further efforts. Also, the possibility of modulating group rather than individual factors appears intriguing and implies great potential not only for application but even for deepening our understanding of evolutionary processes.

Summa summarum, the thesis certainly encouraged my fascination with the staggering diversity of life and I believe it provides valuable insights into the emergence and function of phenotypic variation in the astounding grey partridge, whose rise and fall has been so closely tied to human civilization.

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